Discoidin domain receptor 2: a promising target for novel treatment of fibrosis

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Human diseases resulting in fibrosis still remain a major health problem all over the world. Fibrosis occurs when wounds can no longer properly heal resulting in excessive scarring which can impair function of affected organs. Despite many studies, a lack of knowledge of the processes involving formation of fibrotic tissue and its pathogenesis still exists in addition to the need for novel therapeutic targets. One receptor in particular has been brought to the attention recently as a potential new target, namely discoidin domain receptor 2 (DDR2). In this thesis, the role of DDR2 in fibrosis was assessed by performing literature research. The structure, function, substrate specificity, downstream signaling effects and expression role in fibrosis of DDR2 and its potential as a therapeutic were investigated. DDR2 was found to be a key mediator of collagen deposition due to its ability to influence collagen type I in different ways. DDR2 was also found to be a key mediator of the behaviour of fibroblasts during fibrosis, regulating proliferation, migration, cell adhesion, ECM remodeling and apoptosis. Finally, the expression of DDR2 was found to display differences in progression of fibrosis among organs. In conclusion, DDR2 makes a promising, yet complex target for novel therapies regarding fibrosis.

Keywords: *fibrosis, ECM, wound healing, collagen, DDR2*

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Introduction

Human diseases resulting in fibrosis still remain a major health problem all over the world. One of the main reasons this problem exists is that it affects a large number of people. This can be declared by the variety of diseases that can result in fibrogenesis. These diseases can be systemic diseases such as sclerodermatous graft versus host disease and systemic sclerosis, or organ-specific disorders like cardiac or liver fibrosis (Rosenbloom et al., 2017). These are examples of diseases that can cause damage to tissue when left untreated or severely developed. When tissue is damaged, the body will try to repair it by initiating the wound healing process. Wound healing is a very complicated but well regulated process that is intended to maintain the protective characteristics of the skin (Han & Ceilley, 2017). This process naturally also applies to internal tissue damage. Diseases may affect wound healing causing it to not succeed entirely or even fail, resulting in wounds that are chronic and cannot be healed (Han & Ceilley, 2017). When wounds can no longer properly heal excessive scarring can occur, resulting in fibrotic tissue that impairs function of affected organs (Rosenbloom et al., 2017). In some organs that can suffer from fibrogenesis due to injury, like the liver, fibrosis is not even the end stage of the disease, pointing out the seriousness of the problem even more (Toosi, 2015). Liver fibrosis can namely develop even further to a final pathological stage called liver cirrhosis, which is even harder to treat and cure (Zhou et al., 2014).

Despite many studies, a lack of knowledge of the processes involving formation of fibrotic tissue and its pathogenesis still exists in addition to the need for useful biomarkers and adequate remedial therapeutic agents (Rosenbloom et al., 2017). It is therefore necessary to explore new tactics and potential new agents targeting any component of the mechanism behind fibrosis. For example, many studies have found that myofibroblasts, in active state, are guilty of substituting normal tissue for fibrotic tissue (Rosenbloom et al., 2017). Myofibroblasts seem to already be targets in addition to related mechanisms and signaling pathways for recent research regarding chronic liver diseases (Parola & Pinzani, 2019). Recent renal fibrosis research also seems to include myofibroblasts as targets as well as substances excreted by epithelial cells (Humphreys, 2018). Another method includes focussing on the causes of fibrosis, therefore preventing fibrogenesis, and substituting fibrotic tissue for functional cells (Park et al., 2019). This approach remains important to keep in mind, especially for organs with very complex cells that are hard to culture or repair, like the heart or kidneys.

In addition to focussing on what cells play an important role in the formation of fibrosis, the molecular mechanism behind it can also be looked into. One receptor in particular has been brought to the attention recently, namely discoidin domain receptor 2 (DDR2). DDR2 has been found to be related to pathological scarring of various organs and therefore being a possible key player in the molecular mechanism behind fibrosis (DeLeon-Pennell, 2016). In this thesis literature research is performed to investigate the role of this seemingly novel player, DDR2, in relation to fibrosis.

Research question

What is the role of DDR2 in fibrosis?

What is fibrosis?

Wound healing

When a wound is created internally or externally, the body will try to repair it as was mentioned earlier. The wound healing response is set into motion. Wound healing is a crucial process to maintain the characteristics of the tissue (Wang et al., 2018). It involves various kinds of cell populations, the extracellular matrix (ECM) and soluble components like cytokines and growth factors (Velnar et al., 2009). All that affects wound healing can be divided into different kinds of factors: systemic factors (diseases and problematic perfusion), regional factors (artery, vein or brain related problems), local factors (among others infection and low oxygen tension) and other various factors like nutrition, smoking, and radiation exposure (Hunt et al., 2000). The wound healing process involves several phases of which multiple compositions have been represented over time. Seemingly, wound healing involves three main phases: the inflammatory phase, the proliferative phase and the remodeling phase (Özturk & Ermertcan, 2011). The inflammatory phase was seen before as a division into 3 processes, namely coagulation, haemostasis and inflammation respectively (Velnar et al., 2009). Additionally, more phases can be added according to recent research, namely epithelialization, angiogenesis (formation of blood vessels from existing vessels) and scarring (named separately here, as it is often included with the remodeling phase). A process called neovascularization, similar to angiogenesis, was featured for being a crucial part of wound healing. This process was found to have a major effect during the entire wound healing process and can therefore also be kept in mind (Sorg et al., 2017).

Wound healing may be divided into several phases which may seem likely to follow up one after another for the entire wound, when actually different sections of a wound can be at different stages of the healing process at the same time (Richardson, 2004). Nevertheless, the wound healing process is constant and a timeline can be followed. Coagulation starts right after the tissue is injured (Hunt et al., 2000). It features hemostasis and generation of temporary wound matrix meant to close the wound (Reinke & Sorg, 2012). Shortly after coagulation has started, inflammation is initiated (Hunt et al., 2000). The inflammatory phase consists of an early phase featuring recruitment of neutrophils and a late phase featuring monocyte arrival and transformation (Figure 1) (Reinke & Sorg, 2012).

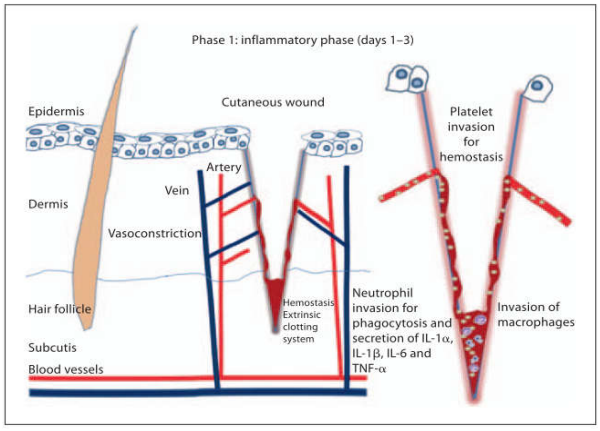

Fig. 1. An overview of the inflammatory phase after injury. Hemostasis and the invasion of inflammatory cells can be seen. *Reinke & Sorg, 2012.*

Afterwards, the proliferative phase follows starting within days after the injury and ensuring a big part of the actual healing (Figure 2) (Hunt et al., 2000). The goal of this phase is generation of granulation tissue, repair of nearby vessels and recovery of the surface of the wound. To achieve this, local fibroblasts immigrate to the wound site through the fibrin network. Then, through capillary sprouting, angiogenesis and neovascularization get activated together with reepithelialization of the edges of the wound. When the granulation tissue is formed, the cells go into apoptosis. The wound can now be seen as mature and is characterised by the absence of blood vessels and cells. During maturation of the wound, ECM components are also likely to change (Reinke & Sorg, 2012). Finally the wound healing process ends with the remodeling phase, which can possibly last up to a year and includes scarring (Figure 3) (Hunt et al., 2000). It can even occur for some wounds to take up to even two years to fully heal, emphasizing the general complexity and the flexibility of the duration of the process (Richardson, 2004). Additionally, even if wounds were to heal completely they would never regain their original vitality (Han & Ceilley, 2017). It seems that it is all the more important that wound healing proceeds successfully, leaving as little damage as possible behind when it's completed, especially since many people all over the world suffer from chronic diseases like diabetes mellitus and peripheral vascular disease which can lead to faulty wound healing (Sorg et al., 2017).

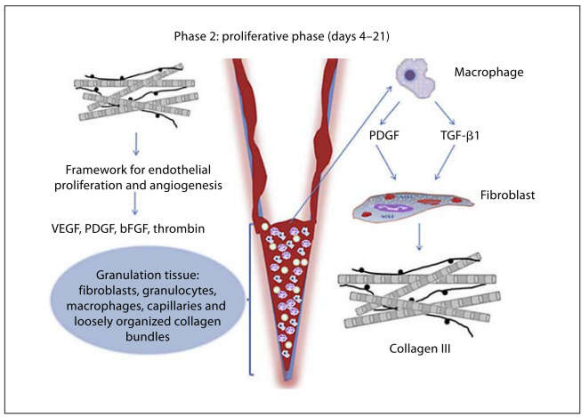

Fig. 2. An overview of the proliferative phase. Thrombus organisation, growth factors secretion, collagen III synthesis and initiation of angiogenesis can be seen. *Reinke & Sorg, 2012.*

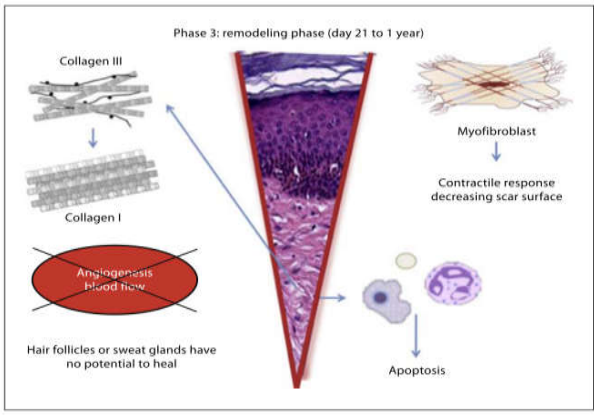

Fig. 3. An overview of the remodeling phase. Regeneration is put to an end, followed by reorganization of connective tissue and contractile response. *Reinke & Sorg, 2012.*

Fibrosis formation

When wound healing occurs in an unnatural manner excessive scar formation can occur, causing fibrosis (Wang et al., 2018). Wounds that won't heal properly will namely become stuck in the inflammatory phase (Özturk & Ermertcan, 2011). Fibrosis coincidentally often occurs as a result of developing a chronic inflammatory disease and is characterised by aggregation of excess extracellular matrix (ECM) components (Wynn & Ramalingam, 2012). This occurs as a result of a disturbed ratio between provocative and suppressive factors (Mutschler, 2012). The cells that are involved in fibrosis formation and deposition of excess ECM are the fibroblasts. Normally, fibroblasts contribute to the processing of

ECM but during tissue repair they transform to myofibroblasts, acquiring phenotypic changes (Figure 4). Myofibroblasts are proliferative and are able to contract, similarly to smooth muscle cells. They contribute to wound repair and die through apoptosis afterwards when a scar is formed. In fibrosis, myofibroblasts don't die, thus residing in the tissue where they contract and dispose of an increased amount of ECM. This leads to growing stiff, thick scars that can be painful. In addition, the excess ECM can impair functioning of the concerning organ. The liver, lungs and kidneys are amongst the organs that can be affected by local myofibroblasts (Darby & Hewitson, 2007). Recent research has also shown the heart to be targeted by myofibroblasts, resulting in myocardial fibrosis. The heart is an example of an organ that has various complex cell types, making it even more difficult to understand the pathology and developing novel therapies (Travers et al., 2016).

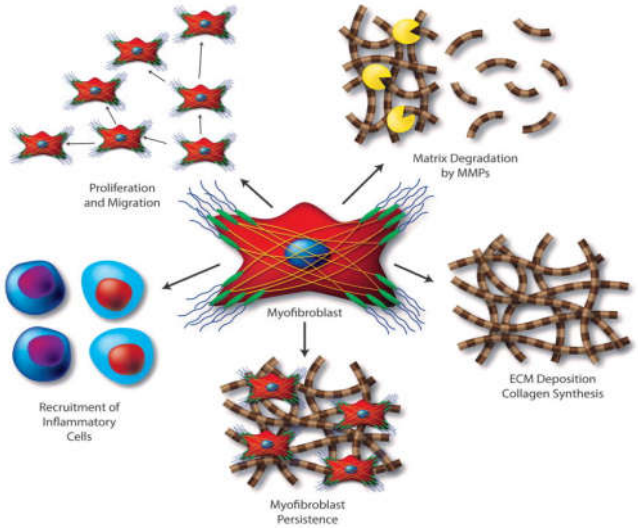

Fig. 4. Functions and characteristics of activated myofibroblasts in the heart (similar to other tissues). In fibrosis, these processes are disturbed. *Travers et al. 2016.*

(Myo)fibroblasts are not the only cell types that contribute to fibrosis. There seems to be crosstalk with macrophages, although loosely defined. Macrophages, in addition to fibroblasts, also seem to play a key role in formation of fibrosis (Witherel et al., 2019). After injury, monocytes migrate to the wound site where they differentiate into macrophages. Macrophages are known to function through the entire wound healing process. Their phenotype and its functions depend on the microenvironment of the wound, therefore changing during the wound healing process. In the early inflammatory phase macrophages execute pro-inflammatory purposes like phagocytosis, antigen presentation and production of growth factors and inflammatory cytokines. Macrophages in this stage are activated classically and can be characterised as M1 phenotype macrophages. In the proliferative phase macrophages trigger proliferation of endothelial, connective and epithelial tissue. This happens both directly and indirectly. Additionally, keratinocytes, endothelial cells and fibroblasts are stimulated as well to activate and achieve reepithelialization, ECM formation and neovascularization. Macrophages are also capable of modifying the ECM composition during angiogenesis as well as during the remodeling phase. This happens through synthesis of ECM molecules and release of degrading enzymes. Macrophages in this stage are activated alternatively and can be characterised as M2 macrophages (Delavary et al., 2011).

If macrophages do not succeed in acquiring the tissue-healing phenotype, they can contribute to formation of fibrotic tissue. Macrophage dysfunction can namely result in sustained inflammation (Smigiel & Parks, 2018). Wound repair no longer occurs normally, characterised by dysregulated production of growth factors and inflammatory signals, defective formation of anti-inflammatory macrophages or disturbed communication between macrophages and various kinds of cells. These cell types include not only fibroblasts and epithelial cells, but also endothelial cells and stem or tissue progenitor cells (Wynn & Vannella, 2016). Macrophages do not only contribute to fibrosis through these actions, they also happen to have the ability to transform into myofibroblasts. Recent research found that bone marrow-derived M2 macrophages apparently have the tendency of transforming to myofibroblasts through regulation via a pathway that involves Smad3 (Wang et al., 2017). Like mentioned before, myofibroblasts are known to contribute significantly to fibrosis formation. Macrophages may be present in all kinds of tissues where they have similar functions regarding tissue repair, but it is possible for differences to occur among these tissues in relation to the priorities and actions of local macrophages, which is important especially in fibrosis. Additionally, besides macrophages and fibroblasts influencing wound repair, the severity and the circumstances in which the wound was formed also affect the wound healing process to a great extent (Oishi & Manabe, 2018).

Scope of the problem

While much about fibrosis is already known, even more remains still unknown: a lack of knowledge about the pathogenesis of fibrosis still exists. Fibrotic diseases are considered a big problem worldwide regarding health due to among other reasons the enormous amount of people it affects (Rosenbloom et al., 2017). In addition, the progression of many diseases is associated with fibrosis formation. Ovarian diseases like premature ovarian failure, ovarian chocolate cyst and PCOS are examples of this (Zhou et al., 2017). Not only can fibrosis occur in the ovaries, it can unfortunately occur in many other organs among which the heart, liver, lungs and kidneys (Rosenbloom et al., 2017). Finally, like many diseases fibrosis is a disease for which a cure does not exist (yet), only medication to slow down the progression of the disease and postponement of death. From all of this it can be concluded that a growing need for novel therapies is inevitable.

The role of collagen in fibrotic tissue

Function of collagen

Collagens are part of one of the many protein families that are represented by the ECM and are known to be most prominently present (Gelse et al., 2003). This protein family is very complex and has an exceptional diversity in function, molecular and supramolecular organisation and tissue distribution. Many types exist, but they all have one characteristic in common: having one triple helical domain or more (Ricard-Blum & Ruggiero, 2005). The triple helical domain consists of three alpha chains which in turn consist of repeating peptide triplets of glycine-X-Y. Any amino acid can be used for X and Y, although proline and hydroxyproline occur the most often. So-called 'non-collagenous domains' also exist, which often contain peptide modules that can be recognized. Similar peptide modules have also been discovered in other molecules of the ECM (Gordon & Hahn, 2010). Cells express receptors which allow collagens to interact with them in a direct way by recognition and binding of the triple helical domains. Cell-collagen interactions can also be carried out indirectly and are therefore often negotiated by matrix glycoproteins (Heino, 2007). Through these interactions, collagens can regulate differentiation, proliferation and migration. Some collagens even carry out specific biological functions because of strictly controlled tissue distribution (Ricard-Blum, 2011). Collagens are also able to assemble into diverse supramolecular structures. This assembly depends on communication with other matrix molecules and cellular components (Mienaltowski & Birk, 2014). Without it, tissues cannot function properly because they are dependent on the correct incorporation of these assembled supramolecular structures into the matrix (Gordon & Hahn, 2010).

Collagens are involved in the wound healing process as well. They are produced by fibroblasts and contribute to wound healing, playing a role in all phases (Fleck & Simman, 2011). The production, organization and distribution is balanced. Within the ECM, this balance is maintained through degrading collagen and remodeling it afterwards (Coelho & McCulloch, 2016). Here, they are involved in establishing structure and assisting in maintenance of tissue shape, organization and mechanical characteristics (Ricard-Blum, 2011). They contribute for example to the formation of basement membranes and a variety of other structures that the ECM contains. They also play a major role in synthesis of fibrillar and microfibrillar networks of the ECM (Gelse et al., 2003). These actions are important because the wound healing response after tissue injury is meant to restore the function and structure of the tissue by creating new ECM (Coelho & McCulloch, 2016). Additionally, they influence migration of cells through stimulation and promote formation of new tissue. Their chemotactic abilities influence fibroblasts which makes it possible to create an environment that favors healing (Fleck & Simman, 2011).

When wound healing fails, collagen remodeling is dysregulated. The balance is disturbed: more collagen is deposed than degraded. This is one of the key characteristics of fibrosis (Coelho & McCulloch, 2016). The composition of the ECM and the collagen types it contains change drastically throughout the disease course of fibrosis. Collagens might namely be able to relocate as a result of fibrosis. This alone can possibly lead to a negative change in function. It is important for collagens to carry out the function they

are originally meant to in order to successfully accomplish wound healing. A change in their function can lead to altered cell function, production of ECM, proliferation and cell fate, therefore contributing to fibrosis. The role of collagens will remain an important aspect in the assessment of fibrosis (Karsdal et al., 2017).

Collagen types in fibrotic tissue

Since fibrosis is mainly characterized by dense ECM, which basically consists of collagen, it might be interesting to look into what types of collagen are involved in fibrosis. Generally, the following classes of collagen can be distinguished:

Class of collagen	Types included in class
Fibril-forming collagens	I, II, III, V, XI, XXVI, XXVII
Fibril-associated collagens that have interrupted triple helices	IX, XII, XIV, XVI, XIX, XX, XXI, XXII, XXIV
Network-forming collagens	IV, VIII, X
Membrane anchored collagens	XIII, XVII, XXIII, XXV
Interconnecting collagens, microfibrils	VI, VII, XV
Short (developmental) collagens	XXVI, XXVIII

Table 1. An overview of how collagens are divided into classes and what types each class contains. *Karsdal et al. 2017*.

As mentioned before, (myo)fibroblasts are cells that mainly produce ECM. In all types of fibrosis they are known to produce excessive ECM, making them an important starting point in identifying the collagen types involved in fibrosis. Fibroblasts are known to mainly produce fibril-forming collagens type I, III and V, fibril-associated collagens (with interrupted triple helices) type XII or XIV and interconnecting collagens/microfibrils type VI. In addition, they also produce network-forming collagen type IV. Collagen type I and III are the majority of what the ECM consists of next to fibronectin (Karsdal et al., 2017). Collagen type V is found in thick fibrils and is also part of the ECM, although minor. It contributes to remodeling of the ECM when degraded by metalloproteinases and gelatinases. Collagen type V is also able to establish interaction with structural proteins and matrix collagens through which they maintain the structural integrity of the tissue (Mak et al., 2016). Despite all its important functions, collagen type V is only a minor fraction of the entire collagen production repertoire of fibroblasts. Collagen types VI, XII and XIV are also produced in small amounts, however, they appear to be very relevant modulators because of their presence on the surface of thick collagen fibrils (composed of collagen type I, III and V). They regulate communication with cells and ECM components and mediate fibril diameters. Finally, collagen type IV contributes to the basement membrane, although the purpose of this is not yet known. In addition, it also debilitates migration of fibroblasts by influencing their microenvironment (Karsdal et al., 2017).

Apparently various collagen types have functions that are normally involved with the ECM, making them potential culprits in relation to fibrosis. Now that we know more about their functions, it is important to narrow down the number of collagen types that match this accusation in order to explore possible new targets.

Briefly, a few collagen types are known to be related to regenerative processes and the progression of fibrosis. Some of these collagen types are necessary for the repair response, whereas other collagen types contribute directly to fibrosis. Collagen type IV, XV and XVIII make up the majority of the basement membrane matrix, which is repaired by specialized epithelial cells after injury. Collagen type I, III and V make up the interstitial matrix. When the interstitial matrix is damaged, the gaps that are formed are repaired by fibroblasts (Karsdal et al., 2017). Since it was concluded earlier that fibroblasts are involved majorly in the pathology of fibrosis, the collagen types of interest can be narrowed down to type I, III and V, given the information that was just obtained. Additionally, these collagen types of interest can be narrowed down even further, since fibrosis is mainly characterized by excessive ECM deposition and the ECM on its turn mainly consists of collagen type I and III as was described earlier (Karsdal et al., 2017). In conclusion, collagen types I and III will be further looked into, exploring the receptors they correspond to.

Collagen receptors

Collagens are released in the ECM where they can fulfill their function. Many of these functions acquire communication between collagen and other components of the ECM. This communication can be realized through interactions via various receptor families (Ricard-Blum, 2011). Some of these families include cell surface collagen receptors that are able to recognize the triple helical domain of collagens. Overall, they mediate a lot of biological processes like immune function, cell migration and hemostasis (Leitinger, 2011). Leukocyte-associated immunoglobulin-like receptor 1, members of the mannose receptor family, glycoprotein VI, integrins and discoidin domain receptors are all cell surface receptors that can recognize collagen (Leitinger & Hohenester, 2007). They are all structurally different, even though they can all be categorized as cell surface collagen receptors (Leitinger, 2011). Leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) regulates inhibition of immune cells. LAIR-1 can be bound by a collagen type XVII but also by collagen type I and III with minor affinity (Leitinger & Hohenester, 2007). It recognizes collagens by their GPO motif. In a more recent study, LAIR-1 was also found to be able to bind collagen type II, XIII, XVII and XXIII (Ricard-Blum, 2011). It is thought that collagen might function as an inhibitor for LAIR-1. Some members of the mannose receptor family, namely mannose receptor (MR) and Endo180, were also found to be able to bind collagen. Endo180 plays a major role in uptake of collagen. When fibroblasts miss Endo180, they are no longer able to incorporate collagen, causing them to adhere and migrate aberrantly on collagen matrices. Interestingly, MR was found to bind and incorporate collagen (Leitinger & Hohenester, 2007). Ligands of Endo180 are collagen type IV and fibril-forming collagens. Glycoprotein VI receptor (GPIV) is present on platelets and is also able to bind collagens by recognition of their GPO motif (Ricard-Blum, 2011). This receptor plays a major role in vascular repair, creating a hemostatic plug after a vessel is damaged. When a collagen

ligand binds GPVI, aggregation mediators are released and platelet integrins are activated. Platelets contain various collagen receptors, making it hard to determine collagen specificity. However, collagen type I and III ligands are known agonists, whereas denatured or soluble collagens appear to be inactive (Leitinger & Hohenester, 2007). Integrins are cell-adhesion receptors that function without intrinsic kinase signaling (Ricard-Blum, 2011). When bound, they maintain cell adhesion to the ECM. It was also found that one member of the integrin family, α1β1, plays a role in fibroblast proliferation. The structure behind recognition of collagens by integrins is well understood, however, the mechanisms behind it are not so well understood (Leitinger & Hohenester, 2007). Discoidin domain receptors (DDRs) are receptors that can bind, among others, collagens type I, II and III and when bound, display tyrosine kinase activities (Ricard-Blum, 2011). DDR1 and DDR2 have soluble extracellular domains that mediate the deposition of collagen upon binding. They do this by inhibiting fibril formation (Flynn et al., 2010). Additionally, DDR2 is able to shorten the persistence length and influence the Young's modulus of collagen type I fibers (Sivakumar & Agarwal, 2010).

To get back at the collagen types of interest (collagen type I and III), together with the information that was just obtained in relation to collagen receptors and their functions, discoidin domain receptors will be explored further as DDR2 seems a promising target for treating fibrosis.

Discoidin domain receptors

Structure

DDRs can be categorized as transmembrane receptors, which consist of an extracellular component and a cytoplasmic component (Leitinger & Hohenester, 2007). While the extracellular domains of RTKs generally consist of various structures like fibronectin domains or immunoglobulin-like domains, the extracellular component of DDRs consists of a DS domain and a DS-like domain which are structurally unique. The DS domain is named after a protein produced by *D. discoideum*, a slime mold. Furthermore, a high sequence identity exists in the extracellular domains of DDRs, being 51% in DS-like domains and even 59% in DS domains, respectively (Leitinger, 2014). The cytoplasmic component of DDRs consists of a major juxtamembrane (JM) domain and a tyrosine kinase domain with a C-terminus, which is however a characteristic all RTKs possess (Leitinger & Hohenester, 2007). In contrast to the DS and DS-like domains, JM regions are not well conserved among DDRs. Both DDRs also possess N- and O-glycosylation sites, which are predicted. N-glycosylation sites seem to have one site among them for each DDR that is conserved and seemingly dominates. In DDR1 this site is Asn211 and in DDR2, this site is Asn213. For O-glycosylation sites, however, none have been found so far. Unlike DDR2, DDR1 receptors have different isoforms (Figure 5). DDR1 transmembrane and extracellular domains are alike among isoforms, whereas the cytoplasmic component varies. DDR1 has five isoforms, namely DDR1a, DDR1b, DDR1c (not shown in Figure 5), DDR1d and DDR1e. Only three DDR1 isoforms (a, b and c) are operative receptors. DDR1 isoforms d and e are truncated proteins that lack operative kinase domains, missing sections of the JM region and the binding site for ATP or even the complete kinase domain.

When co-expressed with full-length receptors, they might be able to mediate DDR1-dependent signaling, although proof for this theory is still lacking (Leitinger, 2014).

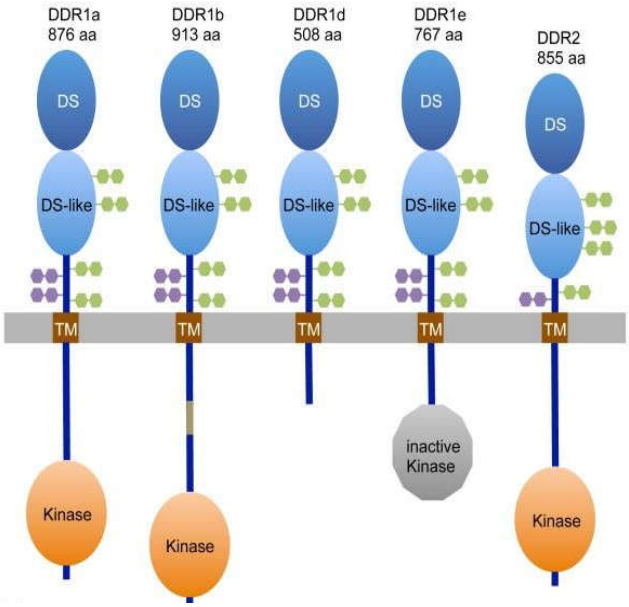

Fig. 5. Schematic overview of DDR1 and DDR2 structures. Top to bottom: DDRs consist extracellularly of a DS domain (N-terminal), a DS-like domain and a JM region. In the cytoplasm, another JM region, the tyrosine kinase and a relatively short C-terminal tail exist. Additionally, the grey bar represents the plasma membrane and the green and purple symbols are predicted N- and O-glycosylation sites. Furthermore, DDR1 isoforms a, b, d and e are shown. In beige, the 37 additional amino acids of the JM region of DDR1b are shown (not shown for other isoforms). *Leitinger. 2014.*

Function

DDRs are collagen binding receptors. DDRs bind various collagen types and display tyrosine kinase activities when bound (Leitinger, 2014). They function without depending on β1 integrins (Leitinger & Hohenester, 2007). Like mentioned before, DDRs have soluble extracellular domains that possess the ability to mediate deposition of collagen in the ECM, which is achieved by inhibition of the formation of fibrils (Ricard-Blum, 2011). Therefore, DDRs are quite different from other receptor tyrosine kinases (RTKs) since they require activation by ECM components while other RTKs require activation by growth factors. DDRs are expressed in matured tissues as well as during development. Where DDR1 is expressed mostly in leukocytes and epithelial cells, DDR2 is expressed in mesenchymal cells (Leitinger & Hohonester, 2007). Furthermore, high levels of DDR1 mRNA were found in the spleen, brain, kidneys, placenta and lungs, whereas high levels of DDR2 mRNA were found in the heart and skeletal muscles, but also in the lungs and kidneys just like DDR1 (Leitinger, 2014). In addition to regulation of collagen deposition, DDRs are also able to affect developmental processes and mediate other processes like proliferation, migration, cell adhesion and ECM remodeling. The latter is realized by maintaining control over the activity and expression of matrix metalloproteinases (Leitinger & Hohonester, 2007). Adhesion and migration are influenced by both DDRs, whereas DDR2 is involved in wound healing and DDR1 in immune responses. It has also become clear lately that DDRs might also contribute to tissue regeneration and homeostasis, since DDR2 affects dermal fibroblasts during wound healing (Leitinger, 2014). It seems like a strong possibility that aberrant DDR2 functioning might play a role in fibrosis, given that normal functioning is involved in the wound healing process.

DDR2: relationship to collagen and fibrosis

Substrate specificity of DDR2

DDRs are known for their unique ability (among RTKs) to bind collagens and their specificity for various collagen types. The DS domain of DDRs is important for the binding of these collagens. Collagen is only able to bind DDR dimers, which are already present on the membrane of the cell (Leitinger & Hohenester, 2007). It is thought that DDR dimers are formed during biosynthesis (Leitinger, 2014). This is in contradiction with other RTKs since dimerisation in other RTKs happens upon ligand binding. It is suggested that for collagens to be recognized, a unique composition of the DS domains is needed within the DDR dimers. Additionally, in order to activate the DDR the triple helical domain within collagens is needed for recognition by the DS domain, since DDRs are not able to bind denatured collagens. DDRs can be activated by various fibril-related collagens as well as collagens that are not fibril-related. DDR2 even has its own preference regarding binding of certain collagen types, namely collagen type II and X (Leitinger & Hohenester, 2007). In a later study, collagen type I and III were also found to be ligands of DDR2 (together with type II as well). In addition, the DDR2 binding site for collagen type I, II and III was found to be a GVMGFO motif (Ricard-Blum, 2011). The DS domain contains an amphiphilic trench on top that is able to recognize the GVMGFO motif. Since DDRs are activated in a slow manner, it is suggested that the supramolecular collagen structure is not needed for activation. Activation of the DDR2 DS domain can be blocked by inhibiting the DS domain allosterically with monoclonal antibodies. Finally, it is suggested that binding of collagen to DDR2 leads to conformational dimeric changes that may result in larger, new structures: DDR clusters (Carafoli & Hohenester, 2013).

Downstream effects of collagen binding of DDR2

When a ligand binds to an RTK, in this case DDR2, the receptor is autophosphorylated. Interestingly, this process is very slow, but sustained in DDRs in comparison with other RTKs (Leitinger & Hohenester, 2007). Where the activation of other RTKs takes only a few seconds to minutes, DDR activation through collagen can take up to hours and can still be detected days after activation (Leitinger, 2014). When collagen binds to DDR2, DDR2 gets activated. The activation of DDR2 is realized by Src, which phosphorylates certain tyrosines that are located in the activation loop of the cytoplasmic tyrosine kinase domain of DDR2 (Carafoli & Hohenester, 2013). Tyr684 and Tyr813 were found to be among these phosphorylated tyrosines. After phosphorylation, these regions can function as a docking site where signaling molecules can bind. For instance, adaptor ShcA is a signaling molecule that can bind on a docking site called Tyr471. Unfortunately, other adaptor molecules like ShcA that establish interaction with DDR2, activated through collagen binding, have not been found yet. One study discovered various potential DDR2 signaling molecules, including PIK3C2A, Src family kinase Lyn, Nck1, PLCL2 and SHP-2, however, experimental validation to support the interaction between these signaling molecules and DDR2 phosphorylated tyrosines is still lacking (Iwai et al., 2013). The signaling pathways involved with DDR activation are unfortunately not entirely understood. Even though our knowledge of DDR signaling

pathways is not complete, certain parts of DDR signaling can already be explained. For instance, one of the signaling pathways that DDRs might be involved with is the mitogen-activated protein (MAP) kinase pathway. This pathway can be activated by DDRs via various members of the MAP kinase family. This way, DDR2 activates MMP-13 in chondrocytes by using p39 MAP kinase and ERK1/2 and activates IL-12 production by only using p38 MAP kinase. In addition, ERK2 is activated by DDR2 in breast cancer cells. It is also thought that DDR2 is able to activate Runx2, a transcription factor, by using p38 MAP kinase or ERK1/2 during differentiation of osteoblasts. However, since various reports state conflicting results, this issue is yet to be resolved (Leitinger, 2014). Furthermore, NF-kB, phosphoinositide 3-kinase and Janus kinase pathways were also suggested to be involved with DDRs in addition to the MAP kinase pathway (Carafoli & Hohenester, 2013). Interestingly, RTK activated signaling pathways were found to be reciprocally connected or connected with distinct cell surface receptor classes. This implicates that various receptor systems are able to cooperate and mediate a certain outcome together. While it is not evident that DDRs have the ability to cooperate with other RTKs, it was recently found that DDR2 phosphorylation through collagen activation is influenced by the insulin signaling pathway. However, reports explaining the mechanism behind this connection are still lacking. Finally, it was found that DDRs are able to influence integrin-mediated cellular functions and vice versa. An example of this is the cross-talk between collagen-binding integrins and DDR1. DDR-activated signaling pathways can apparently merge with integrin signaling pathways, thereby mediating specific cellular functions. Besides, DDRs are also able to influence integrin activity directly. Despite these novel insights, integrin-mediated cellular functions or pathways being influenced by DDR2 specifically were not mentioned in contrast to DDR1 (Leitinger, 2014).

Expression role of DDR2 in fibrosis

Fibrosis is mainly characterized by a dysbalance in collagen housekeeping: more collagen is deposed than degraded. This majorly concerns fibrillar collagens with collagen type I in particular. Since DDR2 is able to bind fibrillar collagens like collagen type I and III as a ligand, it is important to assess the role of DDR2 in relation to fibrosis (Jia et al., 2018). Moreover, a previous study showed reduced fibrosis and a decrease in the number of active fibroblasts in a collagen type I deficient mouse model, contributing to the importance of DDR2 as a target (Yang et al., 2013). The role of DDR2 in fibrosis is yet to be definitively established since the results of different reports imply organ specific differences (Jia et al., 2018). One study showed an increased tendency of developing liver fibrosis in a DDR2 deficient mouse model (Olaso et al., 2011). Interestingly, another study showed a reduction in angiotensin-induced cardiac fibrosis in a DDR2 knockdown model (George et al., 2016). In addition, a recent study using a DDR2 deficient mouse model found reduced lung fibrosis (Zhao et al., 2016). This finding is supported by expression levels of DDR2 in fibroblasts versus alveolar epithelial cells. DDR2 is highly expressed in fibroblasts compared to alveolar epithelial cells. The expression role of DDR2 might therefore be involved with activation of fibroblasts through collagen binding. This was assessed by a DDR2-null mouse model. First, lung fibrosis was induced using bleomycin. After 21 days, the amount of fibrosis was measured using a trichrome staining of lung tissue sections of DDR2-null and WT mice (Figure 6).

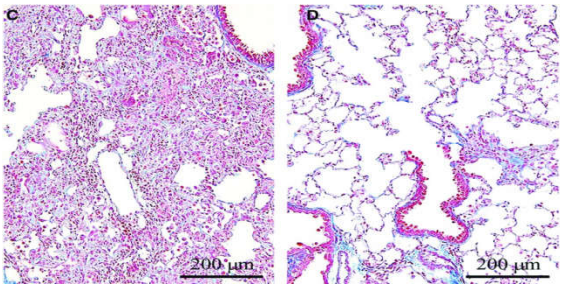

Fig. 6. Trichrome staining 21 days after intratracheal bleomycin induced fibrosis in lung tissue sections (20x) of wild-type (WT) (C) and DDR-null mice (D). The amount of fibrosis is less in DDR2-null mice compared to control. It appears that DDR2-null mice develop less fibrosis over time and are therefore better 'protected' against fibrosis. *Jia et al. 2018.*

Apparently, DDR2-null mice develop less fibrotic tissue over time and are therefore better protected against fibrosis. This implicates that expression of DDR2 and downstream collagen-induced signaling mediates deposition of collagen into the ECM and influences progression of fibrosis. To assess whether DDR2 influences activation of fibroblasts, primary lung fibroblasts from WT and DDR2-null mice were isolated and exposed to TGF-β. Surprisingly, DDR2-null fibroblasts showed reduced levels of activation markers Col1a1, Col1a2 and Col3a1, implying that DDR2 is not involved in fibroblast activation. In addition, DDR2 was not found to be involved with fibroblast proliferation either. Finally, additional experiments showed DDR2 to affect apoptosis of fibroblasts, increasing death of fibroblasts. Thereafter, various signaling pathways involved with DDR2 signaling and fibroblast survival were explored to find a pathway that could potentially enhance apoptosis of fibroblasts via DDR2 regulation even further. Src, XIAP and ERK signaling displayed no differences in phosphorylation between WT and DDR2-null mice. However, Akt signaling, more specifically pThr308-Akt, showed reduced phosphorylation in DDR2-null fibroblasts, implying that enhanced apoptosis of fibroblasts is possible through reduced activation of the Akt signaling pathway. In addition, PDK1 was also found to be reduced in DDR2-null fibroblasts. Since DDR2 regulates PDK1 and PDK1 regulates Akt, the PDK1/Akt pathway is an interesting target pathway to interfere with. This simultaneously contributes to the importance of DDR2 as a potential target in fibrosis (Jia et al., 2018).

Therapeutics

Current pharmacological therapies

Fibrosis is one out of many diseases for which a cure has not been found yet. Despite the lack of a cure, different treatments exist to ease the disease and postpone death. Pirfenidone and nintedanib are characterized as antifibrotic drugs and are currently used to treat fibrosis and most frequently idiopathic pulmonary fibrosis (IPF) (Fujimoto et al., 2016). Both drugs are very beneficial since they are known to slow progression of fibrosis and decrease in mortality (Molina-molina, 2019). Pirfenidone is an anti-inflammatory drug, but hasantioxident and antifibrotic effects as well. The antifibrotic characteristic of pirfenidone is that it has the ability to inhibit several biological processes like production of TGF-β, collagen biosynthesis, proliferation of fibroblasts and production of TNF-α. Despite the many advantages this antifibrotic drug has, it is also inevitable for patients to experience side effects. Common experienced side effects of pirfenidone are gastrointestinal symptoms (nausea, vomiting, dyspepsia and anorexia), neurological (dizziness and headache) and skin related symptoms (photosensitivity and rash),

increased hepatic enzyme levels (can eventually result in aberrant functioning of the liver) and fatigue. The severity of these side effects is characterized as mild or moderate and is dose dependent. Nintedanib is able to inhibit signaling pathways involved with fibroblast, platelet-derived and vascular endothelial growth factor receptors. Common experienced side effects of nintedanib are increased hepatic enzyme levels and diarrhea. It is suggested that a combined therapy, for example of pirfenidone and nintedanib, might achieve better efficacy given the individual benefits of both therapies (Fujimoto et al., 2016). However, one study investigated the combined therapy of pirfenidone and nintedanib and showed no improved efficacy since pirfenidone negatively affected the pharmacokinetics of nintedanib (but not the other way around) (Ogura et al., 2014). Further research is therefore needed to investigate the safety and efficacy of combined therapies (Fujimoto et al., 2016). It can be concluded that while a cure does not exist, the range of current pharmacological therapeutics can be expanded. This is important, especially when organ transplant is the only treatment option left when pharmacological treatment is not sufficient since donor organs are scarce. Furthermore, there is also room for improvement of existing therapeutics, given the severe adverse side effect of nintedanib (increased hepatic enzyme levels leading to potential dysfunctioning of the liver) and the amount of adverse side effects of pirfenidone.

DDR2 as a therapeutic target

Despite already existing treatments for fibrosis, a demand for additional or better pharmacological therapies still exists. DDR2 might be a possible candidate. Research on DDR2 has shown that DDR2 or its related signaling pathway(s) can be targeted to reduce fibrosis. Multiple studies have shown that, despite organ specific differences, DDR2 itself is an important candidate for novel therapies since its expression alone influences the progression of fibrosis. The absence of DDR2 namely results in less collagen deposition and therefore in decreased formation of fibrotic tissue. DDR2 was also found to affect apoptosis of fibroblasts (Jia et al., 2018). Another study found that the DDR2 extracellular domains have the ability to regulate deposition of collagen in the ECM by inhibition of formation of fibrils (Flynn et al., 2010). In addition, DDR2 was also found to influence the Young's modulus and shorten the persistence length of collagen type I fibers (Sivakumar & Agarwal, 2010). These findings also support DDR2 itself as a therapeutic target. One DDR2 related signaling pathway, PDK1/Akt, was also found to be an interesting target pathway to interfere with since it is involved with DDR2 signaling and fibroblast survival. It is suggested that targeting this pathway might enhance apoptosis of fibroblasts (Jia et al., 2018). Finally, some already existing pharmacological therapeutics can also be used to target DDR2. Nintedanib, which is used to treat IPF, is known to inhibit tyrosine kinases and can therefore be used to target DDR2 as well (Jia et al., 2018). Additional tyrosine kinase inhibitors (TKI) can be explored to achieve the same result. An example of this is dasatinib, a TKI used for treatment of chronic myeloid leukaemia (Yurttaş & Eşkazan, 2018). Dasatinib is already known to be compatible as a TKI for DDR2 in treating squamous cell carcinoma of the head and neck (von Mässenhausen et al., 2016). Since dasatinib works specifically on Src family kinases (among which DDR2), its efficacy on DDR2 in relation to fibrosis might be interesting to assess (Yurttaş & Eşkazan, 2018). Moreover, dasatinib is FDA-approved, which makes it easier to test in clinical trials when primary research succeeds (von Mässenhausen et al., 2016).

Conclusion

When assessing the role of DDR2 in fibrosis, multiple aspects of DDR2 and DDRs in general were investigated including structure, function, substrate specificity, expression and potential as a target for a novel treatment. All in all, the following can be concluded: DDR2 is a transmembrane receptor, possessing tyrosine kinase activity, that is able to bind various collagen ligands (Ricard-Blum, 2011). These ligands include collagen type I, II, III and X (Leitinger & Hohenester, 2007)(Ricard-Blum, 2011). The collagen type I ligand is of crucial importance since it is known to be the majority of what the ECM exists of. Again, fibrosis is mainly characterized by excess ECM formation and thereby excessive deposition of collagen type I, making this a very important aspect to take into account regarding the assessment of the role of DDR2 in fibrosis (Karsdal et al., 2017). To support the importance of collagen type I as a ligand of DDR2, it was found that DDR2 is able to mediate collagen deposition through its extracellular domains, the DS and DS-like domain (Ricard-Blum, 2011). Continuing, DDR2 has the ability to inhibit fibril formation, probably via binding of collagen type I given that collagen type I is involved with fibril formation (Flynn et al., 2010)(Karsdal et al., 2017). In addition to this, DDR2 is also able to shorten persistence length and influence the Young's modulus of collagen type I fibers (Sivakumar & Agarwal, 2010). These findings mark DDR2 as a key mediator of collagen deposition, for its ability to influence collagen type I in different ways. In addition to regulation of collagen deposition, DDR2 was also found to affect proliferation, migration, cell adhesion and ECM remodeling through controlling expression of MMPs (Leitinger & Hohenester, 2007). This finding can be supported by the fact that DDR2 is highly expressed on fibroblasts (Jia et al., 2018). It can be assumed that DDR2 is able to modulate all of these processes with regard to fibroblasts. In addition, it was also found that fibroblast survival is dependent on the expression of DDR2, since the absence of DDR2 resulted in apoptosis of fibroblasts (Jia et al., 2018). Fibroblasts are very important cells in the progression of fibrosis, since they are known to accumulate in fibrotic tissue and produce excessive collagen (among which collagen type I) (Jia et al., 2018)(Karsdal et al., 2017). In turn, these findings mark DDR2 as a key mediator of the behaviour of fibroblasts during fibrosis, either worsening the progression of the disease by causing aberrant fibroblast function or reducing fibrosis by inducing apoptosis of fibroblasts. Finally, multiple studies showed that expression of DDR2 displayed differences in progression of fibrosis among organs (Jia et al., 2018). The absence of DDR2 in mice results in either an increased tendency of developing fibrosis or a reduction of fibrosis (Olaso et al., 2011)(George et al., 2016)(Zhao et al., 2016). These findings mark DDR2 as a possible but difficult target for organ fibrosis. Due to organ specific differences, DDR2 cannot be used as a universal therapeutic for fibrosis. Therefore its complexity should be worked out first, so that individual organ specific treatments can be developed. In conclusion, DDR2 makes a promising target for novel therapies since research on DDR2 has come a long way. Meanwhile, additional research is still needed to unravel more about the complexity surrounding DDR2.

Future research

For future research it might be interesting to do data analyses. Information of patients with fibrosis (the type of fibrosis can be picked based on personal interest) can be obtained from various databases. This information can be translated into a list with candidate genes using R (or any other programming software). In R, a manual script can be created and the information can be corrected for variables like age, gender and smoking status (when assessing lung fibrosis). Statistical tests can also be done using the script. When the list of genes is generated, it can be sorted on p-value to see what genes display the highest significance. A selection of genes can be made (for instance, top 10, 20 or 50) to use for STRING analysis. STRING is a program that finds connections between candidate genes based on publications. It also sorts out genes based on their functions. With this approach, possible candidate genes can be found based on data of patients with a fibrotic disease. Since the demand for novel targets is big, this proposition seems suitable.

It might also be interesting to look into other TKIs, as was mentioned earlier. Since TKIs like nintedanib and dasatinib are successfully used, even for multiple diseases, it might be considered likely that other kinds of TKIs are able to achieve similar or better results when applied to fibrosis. In addition, it might also be interesting to look at ways to improve already existing therapeutics like nintedanib or pirfenidone. Similar therapeutics can be discovered by using the program PYMOL. Protein structures with potential ligands can be downloaded from websites like Uniprot and then put into PYMOL. PYMOL can then be used to investigate ligand receptor interactions, together with affinity and the chemical properties of the receptor and the ligands. Structures of nintedanib or pirfenidone can be uploaded to study affinity in order to find a similar but better fitting ligand for the target receptor. Novel structures can also be uploaded so that a whole new ligand (potential drug) can be found.

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