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Fibrosis: A Role for Vitamin D

Noemi Monti[ab]* *and Alessandra Cucina*[cd]

[a] *Department of Experimental Medicine, Sapienza University of Rome, Italy*

[b] *Systems Biology Group Lab*

[c] *Azienda Policlinico Umberto I, 00161 Rome, Italy*

[d] *Department of Surgery "Pietro Valdoni" Sapienza University of Rome, Italy*

***Corresponding author:** Noemi Monti: noemi.monti@uniroma1.it Via A. Scarpa, 16, 00161, Rome

Abstract

Chronic inflammation leads to fibrosis and eventually organ failure. Fibrosis is defined as a wound-healing response that has gone awry. It is featured by excessive production, deposition, and accumulation of extracellular matrix components. The key mediator cells of fibrotic disorders are the myofibroblasts, derived from different precursor cells. Myofibroblasts are responsible of stiff ECM, a hallmark of fibrosis. It is mandatory understanding the molecular pathways contributing to develop the fibrotic tissue to discovery anti-fibrotic therapies. Vitamin D, the precursor of seco-steroid hormone, appears to have anti-fibrotic properties. Vitamin D deficiency may contribute to development of different fibrotic disorders in several organs. It counteracts the pro-fibrotic signals, such as TGF-β1, through several biochemical mechanisms. Counteracting TGF-β1, Vitamin D inhibits myofibroblasts activation and ECM deposition.

Keywords: vitamin D3; fibrosis; IL-6; TGF-β; morphogenesis; epithelial-mesenchymal-transition

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Introduction

An injury in any organ or tissue triggers the repair process, known as wound healing. This process allows the replacement of dead or damaged cells with healthy ones of the same type. Tissue regeneration is the most common outcome of wound healing: connective tissue replacing the normal parenchyma. However, in some circumstances, these processes, can lead to an unwarranted result, as fibrosis. Several acute and chronic stimuli can trigger the repair response; however, if the injurious agents or the damages are not removed, the wound-healing mechanism may "go awry".

This can be ascribed to an increased release of inflammatory mediators and enzymes in the microenvironment. As the inflammatory response becomes chronic, thus, fibrosis occurs. Fibrosis, explained as an "out of control wound-healing response" (Wynn 2007), can eventually lead to organ failure. Fibrosis is characterized by increased accumulation of extracellular matrix (ECM) components, which disrupts the normal tissue architecture. Removing the damaging cause is paramount to avoid the development of a permanent fibrotic tissue. However, identification of fibrosis causative cues is often an uneasy task as well as their removal.

The fibrogenic process

The fibrogenic response can be divided in the following phases: inflammation, proliferation, remodeling and maturation (Rockey et al., 2015). Following an (chemical/physical) injury, the coagulation cascade is the first process that undergoes activation.

Circulating platelets migrate into the wounded area and release a number of growth factors, such as platelet-

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derived growth factor (PDGF) and transforming growth factor β1 (TGF-β1). PDGF is a potent chemoattractant for inflammatory cells, while TGF-β1 is mandatory to stimulate the formation of a provisional ECM by local fibroblasts (Barrientos et al., 2008). The provisional ECM behaves as a scaffold for migration of several inflammatory cells. Concomitantly, damaged epithelial cells also release inflammatory signals, stimulating the proliferation and recruitment of inflammatory cells in the wound. Wounded tissues further produce matrix metalloproteinase's (MMP), which disrupt the basement membrane, thus allowing and facilitating the recruitment of different kind of circulating and inflammatory cells. At this stage, chemical signals such as cytokines and chemokines, recruit endothelial cells to form new blood vessels in the wounded area. Neutrophils and macrophages are the most abundant inflammatory cells at the early stages of process. The former provides an important source of cytokines, activating additional cells to increase the immune response; the latter cleans up tissue debris and dead cells by phagocytosis. Macrophages hold both pro- and anti-fibrotic activity. On the hand, they recruit other inflammatory cells, mainly T-cells, and on the other, they prevent the development of fibrosis eliminating pro-fibrotic factors. Concomitantly, activated T-cells produce pro-fibrotic cytokines, including IL-13 and TGF-β to recruit additional fibroblasts (Li et al., 2006). Fibroblasts, the principal source of ECM components, respond to signals by proliferating and migrating toward to the site of damage. The recruited fibroblasts, in the proliferation phase, rebuild the ECM, replacing the provisional one with fibrillar collagen-rich ECM with higher mechanical strength (Van De Water et al., 2013). Besides producing ECM proteins, fibroblasts are also involved in its maintenance and reabsorption. The reciprocal crosstalk between fibroblasts and macrophages is a key element in fibrosis (Friedman, 2008). Fibroblasts produce pro-fibrotic signals to activate macrophages themselves and in turn, macrophages stimulate the fibroblast to myofibroblast activation (Pakshir and Hinz, 2018). Fibroblasts are quiescent mesenchymal cells, but upon TGF-β1 signal, they are activated into myofibroblasts. Hallmarks of myofibroblasts are high levels of a-smooth muscle actin (α-SMA) expression and a markedly enhanced contractile activity due to the incorporation of α-SMA into stress fibers (Darby et al., 1990), by generating high intracellular tension in the ECM (Hinz et al., 2001). Once activated, myofibroblasts themselves secrete TGF-β, sustaining their own activation by a positive feedback mechanism. The myofibroblasts are rarely found in healthy tissue and they can differentiate from several precursor cells (Gabbiani 2003). Among these cells, tissue-resident fibroblasts are the principal source of myofibroblasts. They can also originate from other sources, including circulating bone marrow-derived fibrocytes (Quan et al., 2006), pericytes (Kida and Duffield, 2011), epithelial and endothelial cells (Carew et al., 2012). Epithelial cells can acquire a myofibroblast phenotype undergoing to a biological process, commonly known as epithelial-mesenchymal transition (EMT). This process triggers biochemical changes in epithelial cells, which lose polarity and acquire mesenchymal features, including enhanced migratory ability, invasiveness, and mainly increased production of ECM components (Kalluri and Weinberg, 2009). It has been recently demonstrated that endothelial cells can also differentiate into myofibroblasts, through an EMT-like process, called endothelial-mesenchymal transition (EndoMT) (Piera-Velazquez et al., 2016). Both EMT and EndoMT can be induced by TGF-β (Pardali 2017). All these precursor cells amplify the pool of myofibroblasts (Hinz et al., 2007). Myofibroblasts synthesize and release elevated amount of matrix components, contributing to the excessive ECM observed in fibrotic diseases. The fibrotic matrix, in the remodeling phase, consists predominantly of fibrillar collagen types I-III (Karsdal et al., 2017), ED-A fibronectin (White et al., 2008), basement membrane collagen type IV, matricellular protein Periostin (PERST) (Kii and Hito, 2017) laminin and other less abundant elements. Myofibroblasts exert mechanical forces on ECM through binding among integrin, ECM components and cytoskeleton filaments (Zhong et al., 1998). Thus, myofibroblasts can remodel both chemical and physical properties of ECM contributing to progression of fibrosis (Hinz, 2016). The mechanical stress of ECM, due to increased tissue stiffness and decreased elasticity, enhances myofibroblasts activation and then progression of fibrosis (F. Klingberg 2013). Therefore, communication in between macrophages and myofibroblasts promotes fibrosis (Hinz, 2009). In the last phase, myofibroblasts stimulate wound contraction, process for the elimination of scar, the re-epithelialization and then the regeneration of the damaged tissue. The apoptosis of inflammatory cells and myofibroblasts is the last fundamental step completing the wound re-

pair process (Mescher 2017). However, if the controlled death fails, this leads to an unbalance between beneficial wound repair and organ fibrosis and it provokes a prolonged activity of macrophages, by activating myofibroblasts, and myofibroblasts by remodeling ECM (Sindrilaru and Scharffetter-Kochanek, 2013). The persistent accumulation and stiffening of ECM supports a positive feedback loop through biomechanical forces by sustaining activation of cells – macrophages and myofibroblasts – beyond their lifetime (Parker et al., 2014). Moreover, during fibrosis, the synthesis of new collagen by myofibroblasts exceeds the rate of its degradation, increasing the amount of matrix (Pardo and Selman, 2006). This is due to the imbalance of collagen turnover, regulated by MMP and their inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), which promote the production instead of the degradation.

Master drivers of fibrotic response

The activated myofibroblasts are considered the main effectors of fibrosis, by producing a large amount of matrix proteins (Hinz et al., 2007). Macrophages and T-cells, instead, release biochemical signals to modulate the fibroblasts activity and the matrix metabolism. Among these signals involved in the process, TGF-β1 is considered the key mediator of the fibrotic response (Stewart et al., 2018). TGF-β belongs to a cytokines family that regulate several physiological processes. There are three different isoforms, TGF-β1, TGF-β2 and TGF-β3, but among these, TGF-β1 is the prevalent and ubiquitously one. The macrophages are responsible to regulate both the secretion and the activation of latent TGF-β1.TGF-β1 is synthesized as latent precursor complex, non-covalently bound to latency-associated protein (LAP) (Robertson et al., 2015). The latent complex is mainly stored in the matrix, covalently cross-linked to ECM proteins (Werb 1997), keeping TGF-β in an inactive form, which cannot interact with its receptors. Several proteases can catalyze the dissociation of LAP from TGF-β1; then, TGF-β1 becomes activated and it can bind to receptors (Murger et al., 1999). Upon binding to transmembrane receptors, TGF-β1 can act through two different signalling pathway: canonical or non-canonical. The canonical signalling pathway, known as Smad-dependent, involves the phosphorylation and activation of Smad2 and Smad3 that form a complex, which subsequently binds to Smad4. The activated complex can translocates to the nucleus and affect the transcription of specific target genes (Hill, 2016). In vivo studies have confirmed the TGF-β/Smad3 involvement in the fibrogenesis because Smad3-null mice became resistant to fibrotic disease (Zhao et al., 2002). However, TGF-β1 can also activate the non-canonical pathway, known as Smad-independent, which involves alternative signaling pathways, including mitogen-activated protein kinases (MAPKs) (Bhowmick et al., 2001), Wnt/β-catenin signaling, phosphatidyl-inositol 3 kinase (PI3K/AKT) (Bakin et al., 2000), p53 and Notch signaling (Zhang, 2009). TGF-β is synthesized and secreted both by macrophages and fibroblasts, thus acting through both paracrine and autocrine way. Upon TGF-β stimulation, fibroblasts are activated in acquiring myofibroblasts phenotype. As mentioned above, TGF-β increases the pool of myofibroblasts also by inducing EMT and EndoMT, respectively, in epithelial and endothelial cells (Xu et al., 2009). TGF-β signaling increases ECM synthesis, deposition, and contraction by myofibroblasts. It mainly enhances expression of collagen types I, III and VI, fibronectin and proteoglycans (Massague, 1990). TGF-β1 also inhibits ECM degradation, by inducing down-regulation of MMP expression and by increasing TIMPs expression, through the Smad3 activity (Yuan and Varga, 2001). Remodeling of ECM is paramount for the progression of fibrosis, as well as for its regression. In skin fibroblasts, TGF-β1 induces the expression of procollagen Lysyl hydroxylase 2 (PLOD2), a gene coding for an important enzyme for the hydroxylation of lysine residues in collagen (van der Slot et al., 2003). Such modification enhances the number of cross-links among pyrydoline residues that stabilize collagen fibrils, making it more difficult to degrade by enzymes (Ricard-Blum et al., 1993). Lysyl oxidase-like 2 (LOXL2) is another important enzyme for the remodeling of ECM components. It catalyzes the deamination of lysine residues of collagen monomers promoting the formation of cross-linkages (Grau-Bove et al., 2015). LOXL2 belongs to the Lysyl oxidase family, composed of five members (LOX and four LOXL variants). The cross-linkages catalyzed by LOXL2are important for collagen stabilization as well as matrix integrity and elasticity. It is noteworthy that LOXL expression and activity are enhanced in hepatic stellate cells (HSCs), involved in liver fibrosis (Liu et al., 2016). These modifications on collagen result in increased matrix stiffness, which activates the myofibroblast via mechanical forces (Ikenaga et al., 2017), thus

allowing the persistence of fibrosis (Hinz, 2016). It has been demonstrated that inhibition of LOXL2 mitigates the progression of liver fibrosis and promotes its resolution (Ikenaga et al., 2017). The expression and activity of LOXL2 are increased by Periostin (PERST) (Kumar et al., 2018), a nonstructural extracellular matrix protein. Periostin can directly interact with ECM components, such as fibronectin, collagen, elastin, and promotes the fibrillogenesis. POSTN stimulates the expression of intra- and extracellular collagen and fibronectin in HSCs, during hepatic fibrosis. Moreover, TGF-β signaling stimulates POSTN and, in turn, POSTN favors the phosphorylation of Smad2/3, even in absence of TGF-β signaling (Kumar et al., 2018).

In addition, other proteins enhance the fibrotic response downstream of TGF-β1 by increasing the contractile phenotype of fibroblasts, as well as by facilitating the release of several ECM components from activated fibroblasts. The ECM is not an inert scaffold, as it is a dynamic regulator of the cell/microenvironment cross talk. Indeed, ECM modulates traffic and activity of several signaling molecules (cytokines, growth factors, etc.) acting upon both cells and their milieu. An important ECM-related pro-fibrotic mediator is the connective tissue growth factor (CTGF), member of a small protein family, localized in the ECM (De Winter et al., 2008). CTGF is a matricellular protein (a non-structural protein found in the ECM) that modulates cell functions through cell-matrix interactions (Chen and Lau, 2009). CTGF is expressed by endothelial cells and by fibroblasts. After being secreted by cells, CTGF interacts with several molecules, mainly cytokines and growth factors. This interaction can affect - positively or negatively - the signal transduction, regulating several processes, such as cell adhesion, migration, ECM deposition and myofibroblasts activation. CTGF plays a central role in tissue remodeling and its expression seems to be correlated with observed clinical behavior. Experimental data on human fibrosis demonstrated that CTGF expression appears associated with the degree of fibrosis (Igarashi et al., 1995). CTGF induces several precursor cells to differentiate into myofibroblasts, including epithelial cells, through EMT process (Lee at al., 2010). Moreover, it increases the expression of collagen type I, fibronectin and integrin in fibroblasts and promotes their deposition and remodeling (Frazer et al., 1996). CTGF can act as an extracellular adapter by binding to TGF-β1 and helping it to bind to its receptors, potentiating its activity 2002). Thus, the overexpression of CTGF enhances the pro-fibrotic response of TGF-β1.Experimental data on transgenic mice with overexpression of CTGF in fibroblasts demonstrated a more accelerated fibrosis development without any other pro-fibrotic stimulus (Sonnylal et al., 2010). Experimental data have highlighted that TGF-β1 induces CTGF expression via Smad-dependent signaling. Smad3 binds the CTGF promoter inducing the myofibroblast differentiation and collagen synthesis (Duncan et al., 1999). Thus, CTGF is considered as a downstream mediator of the effects of TGF-β on fibroblasts (Grotendorst 1997). Moreover, CTGF can also induce the expression of TGF-β itself, triggering a positive feedback loop (Yang et al., 2010). This can contribute to the progression of fibrosis.

Beside TGF-β, other pro-fibrotic and pro-inflammatory cytokines act during fibrosis, especially by T-cells that activate macrophages and fibroblasts (Wynn, 2004) and directly stimulate collagen synthesis in fibroblasts (Oriente et al., 2000) through Smad3/TGF-β-independent mechanisms (Kaviratne et al., 2004). Several findings indicate that cytokines secreted by activated immune cells (T-cells as well as macrophages), such as IL-13, IL-4 and IL-6 can promote an inflammatory/fibrosis prone microenvironment in a TGF-β-independent way (Kaviratne et al., 2004). Studies on cytokine-deficient mice had demonstrated the important link between T-cell response and the development of fibrosis, involving IL-4 and IL-13(Chiaramonte et al., 1999), suggesting that each cytokines likely support a specific role during such process. IL-4, involved mainly in lung fibrosis (Emura et al., 1990), is almost more efficient of TGF-β as pro-fibrotic mediator (Letterio and Roberts, 1998). Fibroblasts express IL-4 Receptors (IL-4R), and upon IL-4 stimulation, they synthesize extracellular matrix components, such as collagen type I, III and fibronectin (Fertin et al. 1991), while in mice treated with IL-4 inhibitors it was demonstrated a reduced deposition of collagen and decreased development of fibrosis (Ong et al., 1998). Other studies showed that IL-13 acts as the effector cytokine of fibrosis when IL-4 is inhibited (Keane et al., 2007). Noticeably, both IL-13 and IL4 converge to the same receptor, which transduce interleukin stimulation through the STAT signaling pathway (Zurawaski et al., 1993). However, quite paradoxically, activation of the same pathway by different effectors (IL4 and IL-13 respectively) fosters the development of different outcomes and the emergence of

diverse types of pulmonary diseases (Zhu et al., 1999; Rankin et al., 1996). This conundrum can partly be explained by the fact that IL-13 may involve alternative pathways usually inaccessible to IL4 (Webb et al., 2003) (Blease et al., 2002). Moreover, IL-13 activates preferentially the TGF-β signaling – by stimulating macrophages to produce latent TGF- β – while upregulating the synthesis of those enzymes (MMP and cathepsin) that activate TGF- β through the cleavage of LAP (Lanone et al. 2002). Indeed, in transgenic mice overexpressing IL-13 and treated with an inhibitor of TGF-β development of fibrosis was markedly reduced (Lee et al., 2001). However, IL-13 can trigger fibrotic pulmonary processes even in the absence of TGF-β/SMAD signaling (Nakao et al., 2000) (Kaviratne M 2004). These controversial findings warrant a convincing explanation that probably require considering the IL-13 mediated involvement of other cytokines in fibrosis development. Noticeably, significant experimental data have been recently collected about the potential pro-fibrotic role sustained by Interleukin-6 (IL-6). IL-6 is a pro-inflammatory and pro-fibrotic cytokine, produced by several cells, including macrophages and T-cells. IL-6 binds to its receptor, IL-6R, and then this complex associates with a second receptor on the cell surface - glycoprotein 130, gp130 (Hibi et al., 1990) - to foster a number of intracellular processes (Rose-John, 2012). IL-6 has higher affinity to IL-6R than gp130, therefore only cells expressing IL-6R can respond to the signal induced by this cytokine (Jostock et al., 2001). However, IL-6R is found only in few cell types, while gp130 is ubiquitously expressed. IL-6R is mainly expressed by hepatocytes and T-cells, limiting the pool of cells able to respond to IL-6 signaling. However, IL-6 can signal through an alternative pathway, known as trans signaling, by which IL-6 recognizes and binds to a soluble form of IL-6R, i.e. sIL-6R, formed by a proteolytic cleavage (Rose-John and Heinrich, 1994). IL-6 has an affinity to sIL-6R comparable to that of IL-6R on the cell membrane. The complex IL-6/sIL-6R can bind to gp130, thus activating trans-signaling-dependent cascades (Taga et al., 1998). IL-6 trans signaling increases the pool of cells that can respond to IL-6. In both pathways, the dimerization of gp130 leads to activation of receptor-associated Janus Kinases (JAKs)(Heinrich et al., 2003), and Signal Transducers and Activators of Transcription (STATs) that, after phosphorylation, can translocate to the cell nucleus where they act as a transcription factors to regulate gene expression(Heinrich et al., 1998). IL-6 plays a role in the development of fibrosis in the lung (Le et al., 2014), by inducing the transformation of fibroblasts into myofibroblasts. Conversely, inhibition of IL-6 trans signaling attenuates pulmonary fibrosis (Le et al. 2014). Levels of IL-6 and sIL-6R are elevated in systemic sclerosis (Hasegawa et al., 1998), correlating with disease severity. In heart, liver, skin and kidney fibrosis (Tanaka et al., 2012), IL-6 induces collagen I expression, both through classical and trans-signaling manner. IL-6 trans signaling increases collagen I expression by activating STAT3 and SMAD3 and by synthesizes Gremlin-1, an antagonist of bone morphogenetic protein (BMP) (O'Reilly S 2014). Over-expression of STAT3 has been observed in tissue of patients with lung fibrosis (O'Donoghue 2012); while, deletion of IL-6 gene results in reduced lung fibrosis in animal models (Saito 2008). In scar tissue, dermal fibroblasts express high levels of gp130 on cell membrane and IL-6 signaling promotes the proliferation of fibroblasts and the increased production of ECM components, including collagen and fibronectin (Ray et al., 2013).

Vitamin D3 biology

Vitamin D is the major regulator of calcium homeostasis and normal bone mineralization in the body (Hoenderop et al., 2005). However, in the last years it became clear that Vitamin D also plays non-calcemic effects modulating other biological functions. Vitamin D, despite its name, is not a vitamin rather it is the important precursor to the seco-steroid hormone, 1α25-dehydroxy-colecalciferol, commonly known as Calcitriol. Calcitriol mediates several biological processes in many tissues. It is obtained from dietary sources or from de novo synthesis. In the skin, the ultraviolet ray's energy converts the substrate 7-dehydrocholesterol to pre-vitamin D3 followed by thermal isomerization to itamin D3 (Dusso and Brown, 1998). The activation metabolism is characterized by two hydroxylation steps, which in turn are principally catalyzed by two P450 cytochrome enzymes (Jones and Prosser, 2014). Vitamin D3 circulates bound to the Vitamin D Binding Protein (VBP), reaching the liver where the first hydroxylation occurs, catalyzed by vitamin D-25hydroxylase (CYP2R1) to yield 25-hydroxyvitamin D3 (25(OH)D), calcidiol (Jones and Prosser, 2014). A further hydroxylation happens in the kidney, where

25(OH)D3 is hydroxylated by another member of the cytochrome P450 family - 1α-hydroxylase (CYP27B1) – to obtain calcitriol (1,25(OH)2D3), i.e. the most active form of Vitamin D3 (Jones and Prosser, 2014). Calcitriol exerts its nuclear effects by binding to the specific Vitamin D Receptor (VDR), a member of the steroid–thyroid–retinoid receptor, which is a superfamily of ligand-activated transcription factors (Christakos et al., 2016). VDR is ubiquitously present into the cytosol of a number of cells. The complex in between Vitamin D3 and its receptor regulates from 3% to 5% of the human genome, via both genomic and non-genomic mechanisms (DeLuca 2004), thus modulating several biological processes (Bouillon et al., 2008). Calcitriol binds to VDR fostering its phosphorylation. It is worth of interest that activated VDR translocates to the nucleus and then it heterodimerizes with Retinoid-X-Receptor (RXR) (Christakos et al., 2003). The RXR is a nuclear receptor activated by 9-cis retinoic acid, playing an important role in regulating retinoid signaling(Heyman et al., 1992).The Calcitriol-VDR-RXR complex recognizes and binds to VDR Element (VDRE) on promoter region of target genes and regulate their expression recruiting transcriptional co-activators or co-repressors(Pike and Meyer, 2012).

Vitamin D and fibrosis: epidemiological and clinical data

Although fibrosis was initially thought to be an irreversible process, experimental data suggest the possibility of resolution of fibrotic diseases (Jun and Lau, 2018). The resolution occurs when the cause of injury is eliminated, but this may not be possible due to the multifactorial feature of this group of disorders. Initially, therapeutic treatments of fibrotic diseases were composed of anti-inflammatory drugs. However, these treatments lack efficacy because they block only the inflammatory cascade, but not the underlying fibrotic response. Therefore, understanding the molecular mechanisms that regulates the fibrotic cascade in every organ provides more specific target for anti-fibrotic therapy. There are three possible therapeutic targets that play a critical role in the resolution of fibrosis: 1) the myofibroblasts; 2) the fibrotic ECM; 3) the cytokines storm. As reported in literature, several compounds have been proposed as possible drugs. Among these, experimental data demonstrated that Vitamin D has anti-fibrotic properties. Epidemiological data have prompted to speculate about a direct relationship between Vitamin D3 deficiency and occurrence of fibrosis-related diseases (Holick, 2007). Beside no unanimous consensus has been reached in identifying the optimal serum levels of Vitamin D3, the cut-off has been set at 10ng/mL while the optimal range of the seco-steroid from 30 to 60 ng/mL. Namely, the role of Vitamin D3 in fibrosis has been largely demonstrated in several organs, in particularly in the liver and kidney, the two organs where vitamin D3 is metabolized. As a proof of concept, supplementation of Vitamin D assumption in patients with chronic diseases, such as kidney fibrosis, results in amelioration of medical condition (Kovesdy et al., 2008). Overall, preliminary clinical data seems to confirm an anti-fibrotic activity of vitamin D3 (Tan et al., 2007).

Mechanisms of Vitamin D in organ fibrosis

The myofibroblast is main target of inhibitory action of 1,25(OH)2D3 during fibrosis processes (Tao et al., 2015). The Vitamin D3 interferes with the pro-fibrotic function of TGF-β1 repressing the expression of collagen in several cell types et al., 2013). Such inhibitory effect of 1,25(OH)2D3 on collagen expression has also been confirmed in experiment conducted on rats. In these experiments, rat hepatic cells treated with Vitamin D3 demonstrated down-regulation of pro-fibrotic genes induced by TGF-β1 (Abramovitch et al., 2011). Calcitriol, bound to VDR/RXR heterodimer, decreases the expression of target genes of TGF-β/Smad3 signaling. Such effect can be exerted through multiple mechanisms, supported by many different scientific evidences. Experimental analysis on hepatic fibrosis revealed the competitive binding of VDR/RXR on the Smad3 Binding Element (SBE), present on the promoter of pro-fibrotic genes. Indeed, through Chip-on-Chip analysis, it has been highlighted that Smad3 and VDR recognize the same binding sites on specific genes (Ding et al., 2013). Maybe, this competition at genomic level is due to the action of specific histone acetyltransferase (HAT) which cause a remodeling of the chromatin, making it in an open status and allowing the docking of the transcription factors (Kouzarides, 2007). For example, p300 histone acetyltransferase is a TGF-β1 target gene and is highly expressed in normal fibroblasts and myofibroblasts during fibrosis (Ghosh and Varga,

2007). Chromatin remodeling induced by p300 allows the opening of the VDR binding sites very close to those Smad3 recognizes. This is a critical step, as opening the chromatin is mandatory for any epigenetic modulation. The binding of VDR upon VDRE causes Smad3 to be dislodged from chromatin causing a blockage of pro-fibrotic genes expression, as both VDRE and SBE compete for the same binding sites. In other experimental studies on hepatic fibrosis, using co-immunoprecipitation analysis, VDR directly interacts with the phosphorylated Smad3, inhibiting the binding of Smad3 to SBE and the subsequent activation of its target genes (Ding et al., 2013). This interaction represses the TGF-β1-induced stimulatory effect on some specific features of the fibrotic process, including collagen release and myofibroblasts activation (Zerr et al., 2015). Myofibroblasts, as mentioned above, can originate from epithelial and endothelial cells, downstream of EMT activation induced by TGF-β1. This pathway is sustained by the activation of several transcription factors, including Smad and Snail (Ikushima and Miyazono, 2010). EMT is a process by which epithelial cells lose their specific markers and acquire mesenchymal traits. Epithelial cells lose cell-cell adhesions and cell-extracellular matrix junctions; in the meantime, they miss their polarity and reorganize the cytoskeleton while promoting a reprogramming of several genes. This transition generates cells with marked motility, able in degrading the extracellular matrix. Such features lead to the acquisition of a migratory and invasive phenotype. Vitamin D is a negative modulator of EMT mechanism, via the transcriptional activity of VDR (Fischer and Agrawal, 2015). Experimental data demonstrated that Vitamin D could attenuate renal fibrosis, by inhibiting the EMT process (Nieto 2011). Specifically, Vitamin D and TGF-β1 have been demonstrated to exert opposite role in respect to EMT, probably accordingly to a reciprocal feedback inhibitory loop. TGF-β, indeed, triggers the expression of VDR and VDR in turn inhibits the EMT process induced by TGF-β. Ricca and coworkers had analyzed the cross talk between the two molecules by treating normal epithelial cells with Calcitriol before, together and after TGF-β addition (Ricca et al., 2019). Calcitriol treatment represses the EMT process, through the up-regulation of epithelial marker, such as E-cadherin, and reduces the cell proliferation rate. An interesting finding is increase in VDR synthesis upon TGF-β1 stimulus. The up-regulation of VDR induced by TGF-β1 is significantly higher than that exerted by calcitriol toward VDR, i.e. its own receptor. TGF-β dependent VDR induction has been demonstrated in different cell lines, being not restricted to few cell types. Probably, the increased expression of VDR is a component of a more complex auto-inhibitory negative feedback loop, enacted by TGF-β upon its synthesis and release (Stroschein et al., 1999). Indeed, among the target genes activated by TGF-β, Smad7, in turn, negatively regulates TGF-β activity (Yan and Chen, 2011). Smad7 competes with Smad2/3 for binding to receptors, thus inhibiting the TGF-β dependent signaling (Yan and Chen, 2011). Notice that Smad7 protein levels are downregulated during kidney fibrosis, while Smad7-deficiency mice are more susceptible to fibrosis (Chung et al, 2009). This data suggests a regulatory role of Smad7 in fibrotic diseases. The auto-inhibitory regulation of TGF-β correlates with data of Ding and coworkers indicating a genomic competition for the binding sites between VDR and Smad3, which immediately increases downstream of the interaction of TGF-β with its receptor. As previously mentioned, the inhibitory action of Vitamin Don EMT occurs only if cells are treated with Vitamin D before (or together) TGF-β addition. Instead, when Vitamin D is added after TGF-β1 stimulation, no significant inhibition on TGF-β-related pathways can be observed and vitamin D result unable in antagonizing the main fibrosis-related molecular features. Vitamin D, in fact, can increase the expression of E-cadherin, and in turn may block EMT, only when is added before or simultaneously with TGF-β1. This finding suggests that, to revert the mesenchymal phenotype, Vitamin D must be already present when EMT is induced. Therefore, the supplementation of Vitamin D seems to have a protecting role against the onset of fibrotic diseases.

Liver cells usually present a low expression of VDR. Recent studies had demonstrated a high expression of the receptor in the hepatic stellate cells (HSCs). Is interesting to observe that the most relevant release of components of ECM produced during hepatic fibrosis can be ascribed to those cells. HSCs, generally, are quiescent and play the role of storage site for Vitamin A (retinoic acid) in the body (Bataller and Brenner, 2011). Following liver injury, HSCs are activated by cytokines and growth factors; then, they differentiate into myofibroblasts, beginning to proliferate, produce cytokines and release the abundant ECM components (Lee and Friedman, 2011). In hepatic fibrosis, Vitamin D represses

the production of collagen by stromal HSCs, through a VDR-mediated mechanism. In addition, Calcitriol suppresses the proliferation of hepatic stellate cells (HSCs) and influences the expression of collagen α I both at transcriptional and translational level, thus inhibiting liver fibrosis (Abramovitch et al., 2011). Indeed, VDR-null mice develop spontaneous liver fibrosis, confirming the important role of the VDR signaling to inhibit the pro-fibrotic transcriptional activity in HSCs (Ding et al., 2013). Moreover, Ding and coworkers, analyzing a mammalian model of liver fibrosis, showed that co-treatment with a Vitamin D analogue caused a reduction in the hallmarks of fibrosis. Specifically, collagen deposition and the expression of pro-fibrotic genes such as COL1A, TIMP1 and TGF-β1 (Ding et al., 2013).Vitamin D counteracts fibrosis inhibiting the expression of collagen I and III and increasing the expression of MMP8, a metalloproteinase essential in the degradation of extracellular matrix in fibrosis. The TGF-β signaling also activates HSCs, which in turn secrete the matricellular proteins, such as CTGF (Liu et al., 2013). The expression of CTGF in hepatic fibrosis increases, but upon Vitamin D treatment, its expression level decreases.

As previously mentioned, Periostin is involved in liver fibrosis, through the stimulation of LOXL2, collagen and fibronectin release by HSCs. It is worth of noting that Calcipotriol, an analog of Vitamin D, can decrease Periostin expression in HSCs (Zhang et al., 2018).

The Vitamin D is also involved in the renal fibrosis. The kidney fibrosis is a hallmark of chronic renal diseases correlating with organ failure. Paricalcitol, a Vitamin D3 analog, decreases interstitial fibrosis, the EMT process and the inflammation response (Tan et al., 2006), by inhibiting both TGF-β expression, ECM components release and the transition of tubular epithelial cells to myofibroblasts (Yang and Liu 2002).In kidney fibrosis, TGF-β and angiotensin II (Ang II) are the major pro-fibrotic drivers (Wolf, 2006). The Renin-Angiotensin System (RAS) is activated in a number of kidney diseases, thus triggering an enhanced release of AngII. The RAS is also activated in lung fibrosis and it is a pathogenic factor in this type of fibrosis (Wang et al., 2015). The RAS includes Angiotensinogen (AGT), Renin and angiotensin-converting enzyme (ACE). AngII, activated by renin and ACE, plays a crucial role in fibrosis, through its receptors, by stimulating the TGF-β pathway, ECM production and driving EMT process to myofibroblasts activation. In the kidney, myofibroblasts differentiate from tubular epithelial cells, which lose the epithelial marker, like E-cadherin, and expressα-SMA 2010). Vitamin D3 plays a protective role, by specifically antagonizing RAS, downstream of VDR activation (Li, 2010). Studies on VDR-null mice have demonstrated that the activation of RAS and the subsequent AngII overexpression play a pivotal role in renal fibrosis. Conversely, treatment with an inhibitor of the receptor of AngII blocks the fibronectin and collagen I expression. On the other hand, Vitamin D suppresses renin and AGT (Yuan et al., 2007), while VDR deletion leads to RAS activation and overproduction of AngII, which induces EMT process and enhances renal fibrosis by stimulating TGF-β synthesis (Li et al., 2002).

Moreover, VDR may regulate (directly or indirectly) other targets involved in renal fibrosis such as Wnt/β-catenin signaling (He et al., 2009). In the adult kidney, the Wnt/β-catenin signaling becomes silent after differentiation, but it can be reactivated upon injury (He et al., 2009). The Wnt signaling pathways comprises a diverse family of secreted lipid-modified signaling glycoproteins that exert their biological function via β-catenin pathway. Wnt transduces the signal through interaction with cell membrane receptor of the Frizzled family and co-receptors, LRP5/6, leading to the de-phosphorylation of β-catenin (Hwang et al., 2009). Upon de-phosphorylation, β-catenin stabilizes and translocates into the nucleus where it binds to and activates several transcription factors, thus regulating the expression of several target genes (Rao and Kühl, 2010). Besides Wnt, other molecular factors can activate β-catenin. A major regulator is the integrin-linked kinase (ILK), induced by TGF-β1, AngII, GSK3β or other pro-fibrotic factors. TGF-β1 can also fosterβ-catenin activation by up-regulating MMP-7 that trigger E-cadherin degradation, thus releasing β-catenin, which is usually linked to E-cadherin behind the cell membrane. When released from its association with E-cadherin, β-catenin can translocate to the nucleus. In addition, Wnt/β-catenin signaling regulates the expression of other critical genes, including fibronectin, MMP-7, fibroblast-specific protein 1 (Fsp1), Snail and components of RAS (Liu, 2011). The activation of these genes suggests a fundamental role of this pathway in modulating fibrosis. The induction of Fibronectin leads up to increased production and deposition of ECM components. Fsp1 is a marker of activated myofibroblasts, produced by resident fibroblasts. Upon tissue injury, β-catenin is highly up re-

gulated in renal tubular epithelial cells and sequentially it induces Snail1 expression. Snail1 is a key EMT-related transcription factor (Yoshino et al., 2007), given that it specifically represses those factors – as E-cadherin – that play a major role in assuring cell-cell adhesion and tissue architecture. Moreover, Snail1 is not only a target of β-catenin signaling but it is in turn regulated by those factors that negatively regulate β-catenin, as GSK3β. Activated Wnt inhibits GSK3βactivity, while allowing the simultaneous release of β-catenin and Snail1. This leads to synergistic effects in promoting EMT (García de Herreros and Baulida, 2012). In view of the role that Wnt/β-catenin signaling plays in kidney fibrosis, is paramount to block that pathway. Noticeably, Vitamin D can inhibit Wnt/β-catenin signaling by three different mechanisms. Firstly, Vitamin D facilitates the reciprocal VDR/β-catenin interaction, preventing the binding of β-catenin to its TCF (T-cell factors) transcriptional factors (Palmer et al., 2001). Second, Vitamin D upregulates the epithelial markers, mainly E-cadherin, thus counteracting directly EMT. Re-expression of E-cadherin allowsβ-catenin cytoplasm translocation and its subsequent interaction with E-cadherin close to the adherens junctions (Palmet et al., 2001). Third, Vitamin D triggers the expression of inhibitors of Wnt signaling, such as Dickkopf (DKK)-1. DKK-1 inhibits the Wnt signaling binding to LRP5/6, i.e. to the receptors of Wnt, thus preventing Wnt/Frizzled/LRP5/6 interaction (Semenov et al., 2001). However, inhibitory activity of Vitamin Dis in turn hampered by Snail1. Indeed, Snail1 represses VDR expression by binding to three E-boxes in the VDR gene promoter. Moreover, Snail1 reduces the half-life of VDR RNA (Palmer et al., 2004).The overexpression of Snail1 in pathological conditions, prevents the expression of E-cadherin and subsequently it blocks the action anti-fibrotic of Vitamin D. This leads to β-catenin translocation to the nucleus and the expression of related target genes. Vitamin D can act as a modulator of immune response, by attenuating inflammatory process and by downregulating the pro-inflammatory cytokine, especially IL-6 (Skrobot et al., 2018). Indeed, a few reports indicate that vitamin D exert an inhibitory role on IL6 release in fibroblasts, probably through the MAPK38 pathway (Nonn et al. 2006). Yet, studies on the relationships between vitamin D and IL6 are still on their infancy and further investigations are warranted.

However, the disparate experimental findings collected until now should be "re-interpreted" according on a very different theoretical perspective in order to gain a more comprehensive appraisal of the physiological role of vitamin D. Overall, data available claim for a relevant morphogenetic role sustained by Calcitriol in modulating the cell-microenvironment cross talk in a number of different tissues, as bone, skin, liver and immune system. To vindicate such a hypothesis, Sonnenschein and coworkers have shown that calcitriol, at physiological doses, affects the mammary gland development contributing to the proper shaping of epithelium (Hasan et al., 2019). In a model in which mechanical forces mediate cell shaping during morphogenesis, the authors observed how mammary cells embedded in type-I collagen matrix manipulate the fibers of collagen to organize the 3D structures, such as ducts and acini (Speroni et al. 2014). The organized collagen fibers constrain the cells on biological processes, including proliferation, apoptosis, and motility, whereas Vitamin D, in turn, constrains the collagen fibers organization, affecting the mechanical forces induced by the cells and ultimately cell population density in a dose-dependent manner. This study highlights that Vitamin D act as a "microenvironment organizer" of morphogenesis that affect both cells and their stroma.

Conclusions

Vitamin D can inhibit and reduce the progression of fibrosis through various mechanisms, as reported in Fig. 1. Principally, vitamin D antagonizes TGF-β dependent pathways by interacting with Smad3, preventing its transcriptional function, or by binding to the binding sites on the target genes promoter, blocking the binding of Smad3 to the same sites. By attenuating the TGF-β pathway, Vitamin D reduces the expression of pro-fibrotic target genes, the transformation of fibroblasts in myofibroblasts and the subsequent EMT. Moreover, vitamin D also inhibits other pro-fibrotic mediators such as LOXL2, POSTN, and IL-6, blocking the excessive accumulation of several components of the extracellular matrix.

By reducing the stiffness of the matrix, Vitamin D interferes with the mechanical forces that activate fibroblasts present in tissue stroma. Overall, these evidences suggest a potential role of Vitamin D as a potential drug for the treatment of fibrotic diseases.

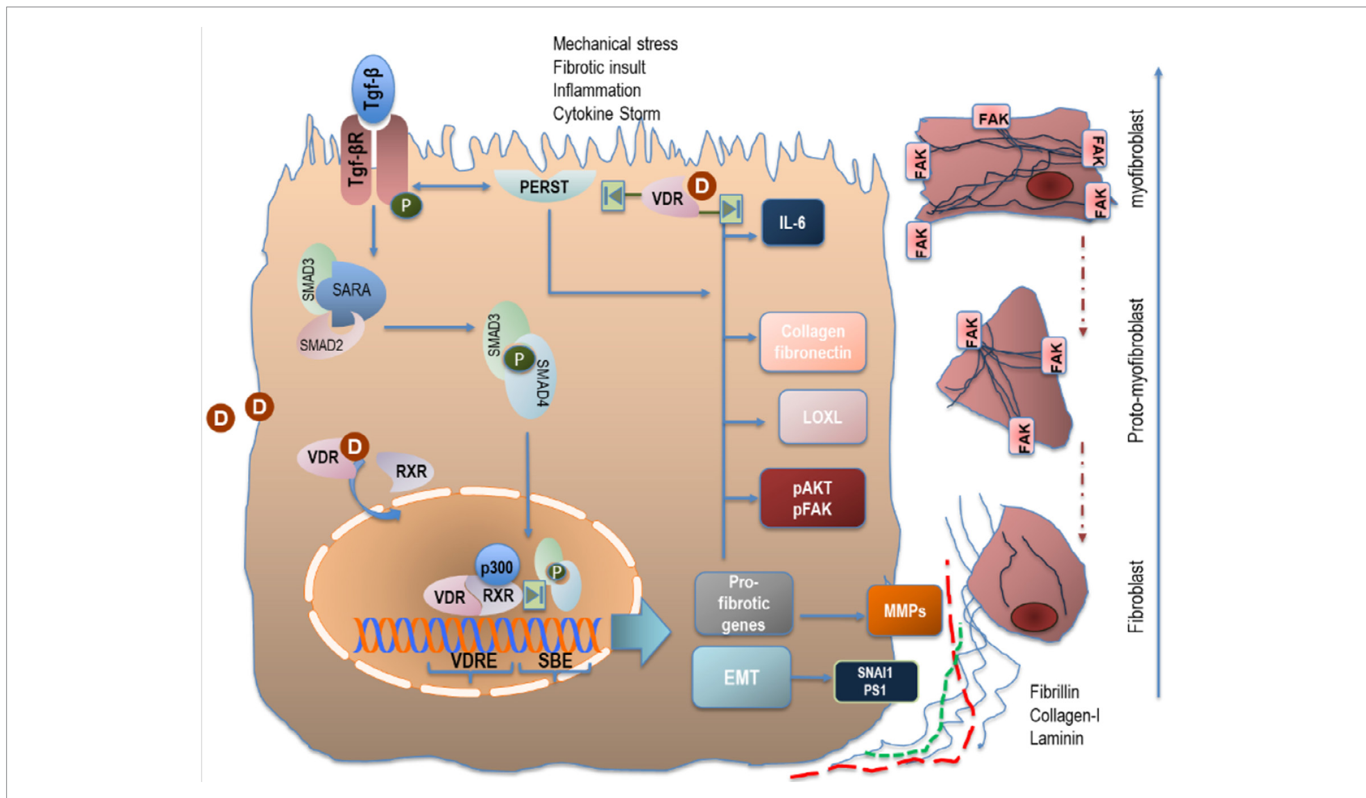

Figure1: Interaction of vitamin D3 with fibrosis-related pathways. Upon TGF-β interaction with its receptor, Smad3/4 complexes translocate into the nucleus and affect the transcription of specific target genes by acting upon the Smad3 Binding Element (SBE), present on the promoter of pro-fibrotic genes. Vitamin D3 compete with Smad elements for binding to the SBE, downstream of the heterodimerization of the VDR/RXR receptors, thus inhibiting the TGF-β-dependent canonical signalling pathway. Vitamin D3 can also directly inhibit Periostin (PERST) and IL-6 release, which are activated upon a variety of pro-fibrotic stimuli. Overall, these pathways promote the transformation of fibroblasts into myofibroblasts, while enacting EMT in numerous cells. Abbreviations: RXR, Retinoid-X-Receptor; Lysyl oxidase-like 2, LOXL2; Vitamin D Receptor, VDR; Epithelial Mesenchymal Transition, EMT.

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