Abstract. The multifaceted involvement of the active vitamin D metabolite 1,25-dihydroxyvitamin D$_3$ (henceforth referred to by the synonyms 1,25(OH)$_2$D$_3$, calcitriol or vitamin D) in blunting the growth of cancer cells is amply recognized. In this review we focused our attention on the cross-talk between 1,25(OH)$_2$D$_3$ and the tumor microenvironment (TME), signaling out stromal cancer-associated fibroblasts (CAFs), the most abundant TME population, as a target for calcitriol anticancer action. In view of the commonality of the phenotypic signature in myofibroblasts, resident in the cancer stroma and in non-neoplastic fibrotic loci, we examined modes of action of vitamin D in non-neoplastic chronic diseases and in cancer to assess mechanistic similarities and divergences. A constant observation was that 1,25(OH)$_2$D$_3$ or synthetic ligands via the active vitamin D receptor (VDR) impede transforming growth factor (TGF)-β/mothers against decapentaplegic homologs (SMADs) signaling in myofibroblasts regardless of the initiating insult. The translational impact of 1,25(OH)$_2$D$_3$ in targetting stromal CAFs is discussed.

Cancer-associated Fibroblasts

For quite a while, stromal fibroblasts surrounding a growing tumor were considered quiescent bystanders and, consequently, their role in cancer development remained largely neglected. This view has been superseded by a vast body of evidence showing that stromal fibroblasts briskly cross-talk with their rogue neighbors and ultimately become partners in crime (1-4). As neoplasia proceeds, fibroblasts are educated by the adjacent cancer cells to foster their growth program and, in their new malevolent vest as activated fibroblasts (hereinafter named myofibroblasts), the non-transformed but pro-tumorigenic cells are properly defined as cancer-associated fibroblasts (CAFs). Of note, CAFs are the most abundant mesenchymal cell population resident in the tumor microenvironment (TME).

We wish at this point to add a note of caution: while a number of studies robustly support the view that heterotypical interactions between CAFs and the tumor cells nurture the neoplastic program (1-4), evidence is available showing that this is not invariably the case as stromal components may act to impede early stage tumorigenesis. In this scenario, the stromal desmoplastic response –the copious secretion by CAFs of collagen fibrils– represents a host defense designed to restrain the growth of the incipient tumor. This view, proposed in early papers by Delinassios working with HeLA cells and human fibroblasts (5, 6) has been recently reviewed in detail (7-9).

A comprehensive discussion of how cancer cells corrupt the naïve stromal fibroblasts and impose a multipronged cross-talk with the neighboring mesenchymal cells to foster their neoplastic growth agenda is beyond the main aim of this review and the interested reader is directed to comprehensive reviews (1-4).

The Myofibroblast in Cancer Stroma and in Non-neoplastic Chronic Diseases: A Compelling Phenotypic Commonality

As mentioned briefly above, one of the principal steps whereby normal stromal fibroblasts acquire the CAF phenotype is their trans-differentiation to myofibroblasts. Notably, we and others (10, 11) have been impressed by the striking similarity in biological behavior and function of the myofibroblast in neoplasia and in a vast range of non-neoplastic chronic diseases that are characterized by extensive
fibrosis, such as hepatic, renal, cardiac fibrosis, to name but a few. Thus, regardless of the initiating insult, myofibroblasts share common markers like the fibroblast-activation protein and α-smooth muscle protein and, importantly, maintain a stable phenotype when severed from the cellular source of their generation (12, 13). This phenotypic durability relies on the reprogramming of their epigenetic landscape (11, 14-17), a well attested genomic signature in CAFs (18-21).

It is worth noticing at this point that when commenting on the shared biological behavior of myofibroblasts we do not refer to functional identity. Indeed, one may tenably argue that subtle phenotypic changes, as yet unidentified, may distinguish between myofibroblasts from disparate tissue origins. A case in point is represented by the epigenetic change in the Ras protein activator like 1 (RASAL-1) gene in kidney, cardiac and hepatic myofibroblasts resident in fibrotic areas (22, 23). RASAL1 is a gene encoding a GAP/GTPase involved in the normal, finely-tuned regulation of KRAS signaling (24). In cells bearing RASAL1 with the changed epigenetic signature, the KRAS gene does act constitutively because of promoter hypermethylation and silencing of the key RASAL-1 GTPase activity. We wish to remark here that the RASAL-1 finding signifies that a stable genomic change in myofibroblasts may occur at regulatory sites of gene expression and not necessarily impinge on the epigenetic landscape of a gene that appears deceptively “wild” and unscathed in function. The RASAL-1 genomic change, found also in epithelial tumors (25, 26), has not been observed (or not searched for) in stromal CAFs. Our preliminary studies have been inconclusive (data not shown).

With the above note of caution in place, the evidence showing similar biological functions in myofibroblasts in fibrotic loci or in cancer stroma is robust, reminding us that cancer is “a wound that never heals” (27), a perpetual lesion showing similar biological functions in myofibroblasts in fibrogenesis has been forcefully demonstrated in 1,25(OH)2D3-null mice that are resilient to experimental fibrosis (40). In addition to its role as a prototypical pro-fibrotic driver, TGB-β induces fibroblast-to-myofibroblast trans-differentiation acting on normal stromal fibroblasts, on fibroblasts surrounding the incipient tumor or in non-neoplastic chronic diseases, such as rheumatoid arthritis, liver fibrosis, kidney fibrosis or systemic sclerosis (41-44), to name but a few.

Vitamin D and Fibrosis

Vitamin D has been extensively studied as an anti-fibrotic agent in non-neoplastic chronic diseases and a number of studies have shown that the myofibroblast is a main target cell of 1,25(OH)2D3 inhibitory action (reviewed in 45, 46). A main and recurring finding has been that 1,25(OH)2D3 interferes with the pervasive pro-fibrotic action of TGF-β: this inhibitory effect is predictable since calcitriol, on its own, represses collagen synthesis in a variety of cells (47, 48).

We have selected liver fibrosis as a paradigm of a chronic disease to gain additional insight into molecular modes of action of 1,25(OH)2D3. A major determinant of liver fibrosis is the reprogramming of quiescent hepatic stellate cells by TGFβ-1/SMADs signaling to a myofibroblast-like phenotype producing excessive extracellular matrix (ECM) components
Hepatic stellate cells are resident, non-parenchymal, perisinusoidal cells in the liver rich in vitamin A esters and endowed with a large range of biological function (49, 50).

In a hallmark paper, Ding et al. (51), using the carbon-tetrachloride mouse model of human liver fibrosis, reported that co-treatment with calcipotriol, a synthetic vitamin D receptor (VDR) agonist, resulted in a reduction in fibrotic scores, collagen deposition and decreased expression of genes involved in the process of fibrogenesis, such as COL1A1, TIMP1 and TGFβ-1. Interestingly, pretreatment with the synthetic vitamin D analogue before administration of the hepatotoxic agent resulted in nearly complete abrogation of fibrosis. The active involvement of VDR in restraining fibrogenesis was strengthened by the observation that VDR knockout mice spontaneously developed hepatic fibrosis.

Exposure of a primary rat hepatic stellate cell line to vitamin D resulted in marked down-regulation of a large number of TGFβ-1-induced pro-fibrotic genes. Importantly, the combination of chromatin immunoprecipitation with high throughput deep-sequencing for identification of genome-wide binding sites in the LX-2 HSC cell line exposed to calcipotriol, vis-à-vis control samples, revealed that the binding sites for VDR and SMAD3 were greatly enhanced and with a nucleosome distance, suggesting a close proximity of the respective cistromes. Indeed, ChiP-on-ChiP analysis revealed that both VDR and SMAD3 were located in the same binding sites. The authors proposed that the VDR/RXR heterodimer interferes with TGFβ-1/SMAD3 transcriptional up-regulation of pro-fibrotic genes by antagonizing SMAD3 binding to their cognate response elements (Figure 1C). This genomic competition was made possible by TGFβ-1-dependent chromatin remodeling, which disclosed a large number of “cryptic” VDR binding sites. This change in chromatin structure was triggered by histone acetylation, a post-translation modification in histone tails that is associated with an open, transcriptionally active chromatin (52). A predominant candidate involved in this process would be acetyltransferase p300, a key transcriptional TGF-β co-activator: of note, p300 is robustly expressed in normal fibroblasts and in myofibroblasts in fibrosis (53, 54). These mechanistic events ultimately provoked a redistribution of genome-wide VDR binding sites (frequently referred to as the VDR cistrome) very close to SMAD3 binding response elements. The overlapping access to DNA and the occupancy by VDR dislodged SMAD3 from the chromatin site and interfered with the pro-fibrotic action of TGFβ-1. Put differently, TGF-β unwittingly brings VDR uncomfortably close to cis-regulatory sequence of SMAD3-responsive genes (Figure 1C).

These results are consistent with previous findings showing that vitamin D exhibits an anti-fibrotic effect in rat hepatic stellate cells via interference with collagen 1α promoter activity (55). In this study, it was also shown that 1,25(OH)2D3 induces an anti-fibrotic phenotype by up-regulating the expression of MMP8, a metalloproteinase that degrades collagen.

In view of Ding et al.’s findings (51), it is pertinent at this point to pause and briefly review what is presently known about the transcriptional activity of VDR. A large body of
evidence supports the view that the active VDR, once bound to 1,25(OH)2D3 or to synthetic vitamin D analogs, translocates to the cell nucleus and forms an obligate heterodimer with the retinoid X receptor (RXR). The VDR/RXR heterodimer, once positioned at vitamin D response element (VDRE), directs the recruitment of nuclear co-activators proteins, e.g. histone acetyltransferases and co-repressors (Figure 1B) (56, 57). Epigenetic changes induce looping of VDR-responsive genomic regions toward the transcription initiation site. The VDR cistrome is highly dynamic and binds to enhancers frequently located in intergenic regions and introns separated by considerable distances from the transcription start site of target genes (57-60).

Acting as a transcription factor, VDR/RXR governs the expression of a large number of genes, and the propensity of VDR to interact with and bind to a vast number of transcription factors to up-regulate or repress gene expression is well-documented (56, 57, 61). Interacting transcription factors include the potent pro-inflammatory nuclear factor-κB (NF-κB) (62) and the growth factor epidermal growth factor (EGF) (63), to name but a few. One of recent findings is the interaction of VDR with nuclear β-catenin (64), a physical connection that impedes the pro-fibrogenetic and oncogenic signaling of the Wnt pathway.

With this background in mind, we can now better appreciate the new mode of VDR action described above in transcriptionally interfering with TGF-β signaling activity, based not on direct binding of the VDR to SMADs but on genomic competition with adjacent transcription factors of SMADs (Figure 1C). As pointed out previously (65), the competitive inhibition of SMADs transcriptional activity by VDR is substantially different from cystromic interactions between transcription factors resulting on mutual exclusion (66).

A number of independent papers reveal that the propensity of VDR and SMADs, in particular SMAD3, to directly or indirectly interact is not restricted to hepatic fibrosis. This was shown in TGF-β-activated skin isolated fibroblasts of patients and in experimental murine models of systemic sclerosis, a chronic disease characterized like hepatic fibrosis by excessive accumulation of ECM components (67). Using reporter assays, target gene analyses and co-immunoprecipitation, these investigators reported that paricalcitol, a synthetic VDR ligand, inhibited fibrogenesis induced by SMAD3 transcriptional gene activation via the binding of VDR to phosphorylated SMAD3, an anti-fibrotic mechanism obviously different from genome competition discussed above (Figure 1B). This repressive action reduced the stimulatory effect of TGF-β on collagen release and myofibroblastic differentiation. Of note, 1,25(OH)2D3 was shown to prevent TGFβ1-dependent pro-fibrotic changes in human primary cardiac fibroblasts (68): the interference with TGF-β downstream signaling appears to result from marked inhibition of SMAD2 phosphorylation due to interaction of calcitriol with cytosolic SMAD2. It remains unclear, however, which molecular requirements underpin the interaction of vitamin D with SMAD2 in the cell cytosol.

While the diverse modes of VDR-SMAD interaction await explanation, possibly reflecting diverse cellular contexts, all of them ultimately lead to the identical final result: down-regulation of TGF-β-induced nuclear SMAD3 transcriptional activity by 1,25(OH)2D3 and the consequent blunting of TGF-β signaling.

The importance of TGF-β, as a main driver of fibrosis, has been emphasized in this review in the context of its cross-talk with vitamin D. However, additional mechanisms and pathways of fibrosis are well-known and the interested readers should peruse excellent reviews on this arresting issue (12, 69).

**Vitamin D and Stromal CAFs**

Fortified from the perusal of these findings, we now address the question whether vitamin D interferes with the action of TGF-β in CAFs via similar mechanistic routes efficiently used in blunting the cytokine activity in non-neoplastic myofibroblasts. This query has been recently experimentally addressed. In a hallmark study, Shermann and colleagues (70) showed that, by targeted remodeling of mouse TME in pancreatic cancer, vitamin D improves drug delivery without interfering with the beneficial action that intact stroma exerts on pancreatic tumors. Notably, calcitriol previously shown to induce quiescence in pancreatic stellate cells, the precursors of pancreatic myofibroblasts (49), reprograms the stromal phenotype to one that is not inflammatory and quiescent.

Interestingly, the assessment of whether VDR/SMAD genomic competition, noted in myofibroblasts in hepatic fibrosis, is also operative in pancreatic stellate cells provided interesting results: calcitriol challenge resulted in decreased SMAD3 binding to promoter regions of pro-fibrotic genes, such as HAS2 (encoding a ECM proteoglycan component) and COL1A1 (encoding the predominant component of collagen), indicating a similar anti-fibrotic action by pancreatic CAFs as shown in myofibroblasts resident in non-neoplastic fibrotic loci. Moreover, in an allograft orthotopic mouse model of pancreatic cancer, intraperitoneal administration of calcitriol in combination with the widely used chemotoxic drug gemcitabine increased the intratumoral concentration of gemcitabine, decreased pancreatic tumor volume and, importantly, markedly increased survival compared to chemotherapy alone. The mechanistic involvement of VDR in inducing fibrosis was again evident in the observation that VDR-null mice showed periacinar and periductal fibrosis. These findings have been reviewed and commented in a number of papers (65, 71).

What is, however, the relevance of the pancreatic cancer studies involving activated VDR and the stroma with respect to other solid malignancies characterized by a strong
effect was markedly enhanced by TGF-β signaling derived from cancer cells. All poor prognosis CRC subtypes were shown to exhibit the same gene program induced by TGF-β in stromal cells. Moreover, CRC patients’-derived organoids relapse in the CRC types. Moreover, CAFs were shown to increase the frequency of tumor-initiating cells and this identified stromal markers that were indicative of disease stromal cells, particularly in CAFs, rather than in epithelial subtypes displaying resistance to therapy and poor diagnosis by Calon et al. (74). These changes obliterate the anti-growth action of the cytokine acting as a tumor suppressor gene at early stages of tumorigenesis (31). Intriguingly, however, TGF-β or SMAD mutant CRC cells retain their capacity of abundant TGF-β production. There is here an interesting conundrum: What is the selective advantage of cancer cells to produce a potent growth factor bereft of the cognate, responsive receptor? It has been shown that opportunistic tumor cells with a disabled TGF-β receptor exploit their own unimpaired synthesis of TGF-β by using the cytokine for the paracrine delivery of signals to CAFs that are endowed with a wild TGF-β receptor and an unimpaired SMAD downstream pathway. In turn, challenged CAFs respond with the production of pro-tumorigenic growth factors and interleukins acting on the cancer cells, a vicious circuitous route that ultimately sustains and reinforces their relentless oncogenic program (75). Importantly, CAFs autonomously synthesize and secrete copious amounts of TGF-β, thus generating an autocrine loop that sustains the fibroblast-myofibroblast trans-differentiation process (76) with expansion of their own population. The reader, eager for additional details pertaining to TGF-β mechanistic involvement in the CAF-driven colonic neoplasia, is directed to a recent excellent review by Calon et al. (77).

In a recent paper, Calon et al. (78) observed that CRC subtypes displaying resistance to therapy and poor diagnosis are characterized by genes expressed predominantly in stromal cells, particularly in CAFs, rather than in epithelial tumor cells. Bioinformatics and immunohistochemical assays identified stromal markers that were indicative of disease relapse in the CRC types. Moreover, CAFs were shown to increase the frequency of tumor-initiating cells and this effect was markedly enhanced by TGF-β signaling derived from cancer cells. All poor prognosis CRC subtypes were shown to exhibit the same gene program induced by TGF-β in stromal cells. Moreover, CRC patients’-derived organoids and xenografts showed that the use of an inhibitor acting on the TGF-β receptor, thus blocking the malevolent cross-talk between colorectal cancer cells and the stromal cytokine described above, resulted in impeding disease progression. Predictably, pharmacological silencing of the TGF-β receptor affected only the stromal cells. In a parallel work, Isella et al. (79) showed that the CRC transcriptome is mostly derived from stromal CAFs.

Notably, likewise in CRC, TGF-β signaling is silenced in a large number of tumor pancreatic cancers, particularly by mutations affecting SMAD 4, the mandatory conveyor of R-SMADs into the cell (80). In these mutant cells, transport of R-SMADs into the nucleus comes to a standstill. A tenable possibility is that pancreatic adenocarcinoma cells devoid of TGF-β signaling communicate with CAFs via cancer-derived TGF-β exploiting the vicious circuitous route experimentally described in CRC.

Having ascertained that TGF-β signaling from CAFs is a main motive in driving CRC, we turn now to 1,25(OH)2D3 and its anticancer effect on CRC. A number of studies have shown that vitamin D is effective in blunting colonic carcinogenesis in animal models (81) and various lines of evidence, but not all, indicate that vitamin D deficiency is associated with increased risk of colonic cancer (82-84). Findings have shown that VDR is down-regulated in a proportion of human colonic carcinomas, thus limiting the use of 1,25(OH)2D3 treatment to adenomatous stages in CRC. A recent paper by Ferrer-Mayorga and colleagues (85) adds new interesting results to this issue. These investigators observed that a high VDR density in CAFs is associated with longer survival in a large cohort of CRC patients independently of its expression in adenocarcinoma cells. Patient-derived colonic CAFs expressed VDR and responded to 1,25(OH)2D3 with the inhibition of CAF pro-migratory effects on cancer cells and of collagen contraction, a major hallmark of myofibroblast activity. Moreover, vitamin D was shown to modulate CAF-global gene expression program inducing a gene signature that afforded a favorable clinical outcome in CRC patients. Cumulatively, these results indicate that 1,25(OH)2D3 exerts protective effects against CRC via the regulation of CAF expression and action and, moreover, suggest that a putative therapeutic action of VDR ligands may be extended to a cohort of patients with CRC at advanced stages of the disease.

Notwithstanding the recurring observation that vitamin D restrains the progression of CRC and the pervasive involvement of TGF-β in this neoplastic process, there is a surprising dearth of studies exploring the possibility that 1,25(OH)2D3 exerts anticancer action by interfering with TGF-β/SMADs signaling in CRC. Thus, the mechanistic “convergence” between the calcitriol and the cytokine shown in fibrosis and in pancreatic cancer remains to be established. However, on the strength of the cumulative results discussed above, we hold the tenable view that one of the main
The recognized commonality in the phenotype of the myofibroblasts and their biological response in tumors and fibrotic loci should be not surprising considering that unresolved, chronic inflammation is the original sin responsible for driving both fibrosis and cancer (86-92).

Neoplastic cells trigger an inflammatory response that builds up a pro-tumorigenic microenvironment and up-regulates the inflammatory profile of stromal fibroblasts (93). In addition, CAFs maintain the inflammatory TME by expressing a frank pro-inflammatory gene signature and by
acting as brisk recruiters of pro-inflammatory cells. Importantly, CAFs produce pro-inflammatory factors that reinforce and maintain stromal inflammation (94-97). Frequently, the same pro-inflammatory cytokines, e.g. IL-6, IL-11, are produced by cancer and stromal cells. Therefore, in a vicious circular train of events, both tumor cells and CAFs contribute to the TME inflammasome (Figure 2A).

One of the pivotal inflammatory transcription factors is NF-kB, a family of dimeric transcription factors expressed in CAFs (96) with a cardinal role in the regulation of immune responses, inflammation and cancer (98). We have mentioned briefly before that 1,25(OH)2D3 inhibits NF-kB activation and signaling via its “messenger VDR” by acting on several components of the multifaceted NF-kB system (62, 99). It is worth noticing that previous works have shown that fibroblasts lacking the vitamin D receptor exhibit increased NF-kB activity (100, 101). Obviously, an additional mechanistic route, whereby vitamin D interferes with CAF pro-tumorigenic purposes, is by blunting their potent NF-kB pro-inflammatory signaling.

While previous studies pertaining to drug-related therapeutic response and resistance were centered on the tumor cell, accruing evidence that TME is intimately involved in promoting the neoplastic process makes, the identification and characterization of drugs interfering with the brisk stroma-tumor dialogue a cardinal aim of translational and clinical oncology. Targeting of stromal TME pro-tumorigenic components, such as CAFs, is presently under intense interrogation.

As outlined previously, ablation of pancreatic stroma is associated with worsening of the cancer progress. However, irrelevant of whether cancer stroma is protective or cancer-promoting, the tumor dense fibrotic desmoplastic reaction may greatly impair the therapeutic efficacy of a drug by limiting or blocking its delivery, thus imposing a serious therapeutic impasse.

The type of therapeutic stromal resistance, which provides a sanctuary for cancer cells from cytotoxic agents, is obviously different from tumor intrinsic or adaptive resistance to chemotherapy and molecularly targeted therapy (102). The active participation of TME in the regulation of therapeutic response in neoplasia has been incisively reviewed (103).

We and others (104-107) hold the tenable opinion that, in contrast to the ablation and loss of stromal components (108), the reprogramming and re-education of specific TME cell populations, such CAFs, are obviously the most logical and less disrupting approaches to selectively modify TME: in this context, vitamin D reprogramming of the pancreatic stroma (70) by promoting the dedifferentiation of hepatic stellate cells is a salient case in point. Another pertinent example is the reprogramming of TME by disruption of the C-C chemokine receptor type 5 (CCR5)-induced homing of regulatory T cells in a mouse pancreatic ductal adenocarcinoma model (109). This inhibitory action, associated with reprograming of TME to support antigen presentation, may improve the efficacy of checkpoint-based immunotherapy.

The evidence for targeting specific TME components, in general, and CAFs, in particular, as a valid approach to an antitumor treatment rests on sound logic: we believe, however, that drug interventions, aimed to the complex and intimate tumor-stroma liaison or to a single stromal cell population, will not lead to tumor banishment but, at best, to restraining and quenching the relentless tumorigenic drive. A combinatorial strategy impinging on both cancer cells and their rogue mesenchymal neighbors should, therefore, be an integral part of treatment protocols focused on interfering with cancer development. In this context, 1,25(OH)2D3, acting on both cancer epithelial cells and stromal TME components, such CAFs, is well-qualified not only as an adjuvant treatment but also as a potential dual benefit drug.

In this review, the active VDR occupies a key mechanistic role acting on the myofibroblast chromatin. A decade ago, we published an editorial (110) on the mechanism of vitamin D. Many unresolved questions raised at that time, such as the functional relationships between VDR and histone post-translational modifications, as well as the temporal order of VDR co-factors recruitment at the VDRE, have been answered. Other questions, to date, await explanation and are the focus of intense investigation.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

References


83 Klampfer L: Vitamin D and colon cancer. World J Gastrointest Oncol 6: 430-437, 2014.


