doi:10.1016/j.jmb.2008.10.060 J. Mol. Biol. (2009) 385, 902–911

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

Regulation of Collagen Fibrillogenesis by Cell-surface Expression of Kinase Dead DDR2

Angela R. Blissett[1], Derek Garbellini[1], Edward P. Calomeni[2], Cosmin Mihai[1], Terry S. Elton[3] and Gunjan Agarwal[1,4]*

[1]*Davis Heart and Lung Research Institute, 473 West 12th Ave. Columbus, OH 43210, USA*

[2]*Department of Pathology, M018 Starling Loving Hall, 320 W. 10th Ave., the Ohio State University Columbus, OH 43210, USA*

[3]*Division of Pharmacology, College of Pharmacy, 217 Parks Hall, 500 West 12th Ave., the Ohio State University, Columbus, OH 43210, USA*

[4]*Biomedical Engineering Department, 270 Bevis Hall, 1080 Carmack Road, the Ohio State University, Columbus, OH 43210, USA*

Received 23 June 2008; received in revised form 6 October 2008; accepted 20 October 2008
Available online 30 October 2008

Edited by M. Moody

The assembly of collagen fibers, the major component of the extracellular matrix (ECM), governs a variety of physiological processes. Collagen fibrillogenesis is a tightly controlled process in which several factors, including collagen binding proteins, have a crucial role. Discoidin domain receptors (DDR1 and DDR2) are receptor tyrosine kinases that bind to and are phosphorylated upon collagen binding. The phosphorylation of DDRs is known to activate matrix metalloproteases, which in turn cleave the ECM. In our earlier studies, we established a novel mechanism of collagen regulation by DDRs; that is, the extracellular domain (ECD) of DDR2, when used as a purified, soluble protein, inhibits collagen fibrillogenesis *in-vitro*. To extend this novel observation, the current study investigates how the DDR2-ECD, when expressed as a membrane-anchored, cell-surface protein, affects collagen fibrillogenesis by cells. We generated a mouse osteoblast cell line that stably expresses a kinase-deficient form of DDR2, termed DDR2/-KD, on its cell surface. Transmission electron microscopy, fluorescence microscopy, and hydroxyproline assays demonstrated that the expression of DDR2/-KD reduced the rate and abundance of collagen deposition and induced significant morphological changes in the resulting fibers. Taken together, our observations extend the functional roles that DDR2 and possibly other membrane-anchored, collagen-binding proteins can play in the regulation of cell adhesion, migration, proliferation and in the remodeling of the extracellular matrix.

© 2008 Elsevier Ltd. All rights reserved.

Keywords: DDR2; collagen; fibrillogenesis; TEM

Introduction

Collagen type I in its mature fibrillar state is the major component of the extracellular matrix (ECM) in most mammalian tissues.[1] Collagen fibers impart mechanical strength to the tissue and interact with cells through cell surface receptors and soluble proteins, which is integral to cell proliferation, migration, survival, attachment and cellular differentiation. The assembly of collagen fibers (fibrillogenesis) is a complex process regulated, in part, by a variety of collagen-binding proteins and other molecules that may directly or indirectly interact with the collagen molecules and fibrils. Several collagen-binding proteins such as decorin[2], lumican[2], cartilage oligomeric matrix protein,[3] fibromodulin,[4] SPARC,[5] and matrilin,[6] etc. have been shown to influence collagen fibrillogenesis. However, almost all these proteins occur as cell-secreted, soluble proteins in the ECM. It is not well understood to what extent collagen-binding proteins anchored to the cell surface affect the assembly of collagen fibrils in the ECM.

The collagen-binding membrane proteins, discoidin domain receptors 1 and 2 (DDR1 and DDR2) belong to the family of receptor tyrosine kinase and

*Corresponding author. E-mail address: agarwal.60@osu.edu.

Abbreviations used: ECM, extracellular matrix; DDR, discoidin domain receptor; ECD, extracellular domain; KD, kinase dead; MMP, matrix metalloprotease; TEM, transmission electron microscopy; HP, hydroxyproline; vWF, von Willebrand factor; IP, immunoprecipitation.

0022-2836/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.

are expressed in a variety of mammalian cells.[7,8] These transmembrane glycoproteins (~125 kDa) have been found to be over-expressed or atypically expressed in several malignancies,[9–12] and regulated in diseases such as atherosclerosis,[13] lymphangioleiomyomatosis,[14] rheumatoid arthritis,[15] and osteoarthritis.[16] DDRs are characterized by three distinct regions:[17] an extracellular domain (ECD), which is responsible for collagen binding, a transmembrane region and an intracellular kinase domain. Binding of collagen(s) to the DDR ECD is known to induce tyrosine phosphorylation of the DDR kinase domain;[7,8] prolonged activation of the DDR kinase domain results in upregulation or activation of matrix metalloproteases (MMPs 1, 2, 9 and 13), which cleave and degrade the collagen fibers in the ECM.[7,14,16]

A second mode of collagen regulation reported earlier by our laboratory shows that the ECD of DDR1 or DDR2 when expressed as a soluble protein can modulate fibrillogenesis of collagen type 1 *in-vitro*.[18,19] In particular, we found that DDR2 ECD delays collagen fibrillogenesis and the collagen fibers formed in the presence of DDR2 ECD were thinner and lacked the native D-periodic banded structure. However, these earlier observations were based mainly on using purified collagen and a soluble form of DDR2 ECD, whereas thus far the DDR2 ECD has only been reported as an integral component of the membrane-anchored, full-length DDR2 receptor.

Therefore, in this study we asked if the expression of DDR2 ECD anchored to the cell surface preserves the capacity to modulate collagen fibrillogenesis for collagen endogenously secreted by the cells. To address this question, we created stably transfected mouse osteoblast cell lines to express a DDR2 isoform, named DDR2/-kinase dead (KD), which resembles the naturally occurring full-length DDR2 except that it lacks the kinase domain. We could thus ensure that our observed effects on collagen morphology and structure would be due only to DDR2 ECD interaction and not through the cleaving action of MMPs, known to be activated upon DDR2 kinase domain activation. Since mouse osteoblasts endogenously secrete collagen, we were able to examine the effects of DDR2/-KD expression on collagen morphology and deposition in the ECM by using techniques such as transmission electron microscopy (TEM) and hydroxyproline (HP) assay. We elucidate how the cell-surface expression of DDR2 ECD plays a major role in regulating the rate of collagen fibrillogenesis and morphology of collagen fibers. Our results demonstrate a novel mechanism of collagen regulation by DDRs and signify the importance of cell-surface-anchored, collagen-binding proteins in regulating collagen fibrillogenesis.

Results

Characterization of DDR2/-KD and stable cell lines

To evaluate the changes in ECM induced by expression of DDR2/-KD, we utilized mouse osteoblasts cells (MC3T3, E1 subgroup-4 clone), which are known to endogenously secrete collagen and generate well defined collagen fibers in their ECM. These cells were used previously to demonstrate that overexpression of lysyl hydroxylase-2b leads to defective collagen fibrillogenesis.[20] Collagen assembly in the ECM of these cells takes one to several weeks; therefore, it was necessary to stably transfect these cells with DDR2/-KD to observe its effect on collagen fibrillogenesis.

Figure 1a shows a representation of the DDR2/-KD construct, which leads to the expression of a truncated DDR2 protein, preserving the extracellular, transmembrane and juxtamembrane regions but

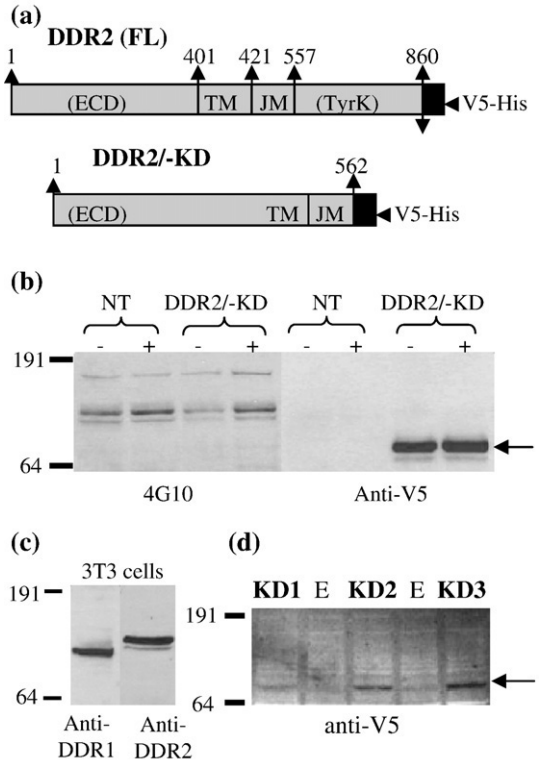

Fig. 1. Creation of stable cell lines expressing the recombinant protein DDR2/-KD. (a) A schematic representation of V5 His-tagged full-length mouse DDR2 and of the V5 His-tagged, membrane-anchored, kinase-deficient DDR2 (DDR2/-KD) transmembrane protein. The extracellular (ECD), transmembrane (TM), juxtamembrane (JM) and tyrosine kinase (TyrK) domains are indicated. The numbers denote the sequence of amino acids in our recombinant proteins. (b) DDR2/-KD does not undergo collagen-induced tyrosine phosphorylation. Following SDS-PAGE, Western blot of whole cell lysates from native (NT) or transiently transfected (DDR2/-KD) HEK293 cells before (–) and after (+) collagen stimulation, were probed using anti-phosphotyrosine (4G10) or anti-V5 antibodies. While the ~125 kDa band indicates phosphorylation of endogenously occurring DDRs, no phosphorylation signal was present for DDR2/-KD (indicated by an arrow). (c) Western blot indicating expression of endogenous DDR1 and DDR2 in 3T3 cells. (d) Three stable MC3T3 cell lines, KD1, KD2, and KD3, were selected for our study, which show increasing levels of DDR2/-KD expression (arrow). E, Empty lane.

lacking the kinase domain. This DDR2/-KD construct is tagged with a V5 and multi-His epitope at its C-terminus. To verify that the DDR2/-KD proteins do not undergo collagen-induced tyrosine phosphorylation, HEK 293 cells were transiently transfected with DDR2/-KD, stimulated with collagen and analyzed by SDS-PAGE and Western blotting with anti-phosphotyrosine antibody. HEK 293 cells, which have an endogenous population of DDR1 and DDR2 (data not shown), show a collagen-induced phosphorylation signal around a mass of 125 kDa. No phosphorylation band was detected for DDR2/-KD protein (expressed ~70 kDa, indicated by an arrow), consistent with the removal

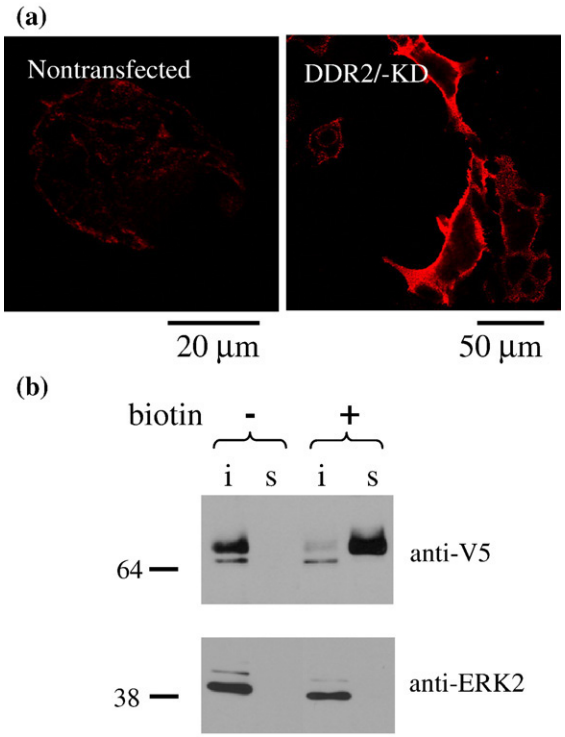

Fig. 2. DDR2/-KD is localized on the plasma membrane. (a) Confocal images of HEK293 cells transiently transfected with DDR2/-KD and immunolabeled with an antibody against the DDR2 ECD; nontransfected cells show a low level of endogenous DDR2, while transfected cells show an increased level of DDR2 ECD on the membrane. (b) Cell surface biotinylation of MC3T3 cells expressing DDR2/-KD construct, demonstrating surface localization of the truncated receptor. Cell samples were biotinylated (as indicated) and surface proteins were precipitated from the lysates with streptavidin-agarose beads (*s* lanes); the supernatant from the pull-down was recovered and probed for intracellular proteins (*i* lanes). Following SDS-PAGE, samples were analyzed by Western blotting with antibodies against V5 tag (DDR2/-KD) or ERK2 as indicated. DDR2/-KD is present mainly on the cell surface, while a lower molecular mass band (probably indicating the immature, nonglycosylated receptor) is found only in the intracellular lane. The intracellular protein ERK2 is present only in the intracellular lane, demonstrating that biotinylation is specific to cell surface proteins. As an additional control, nonbiotinylated samples show that the streptavidin pull down is specific to biotinylated proteins.

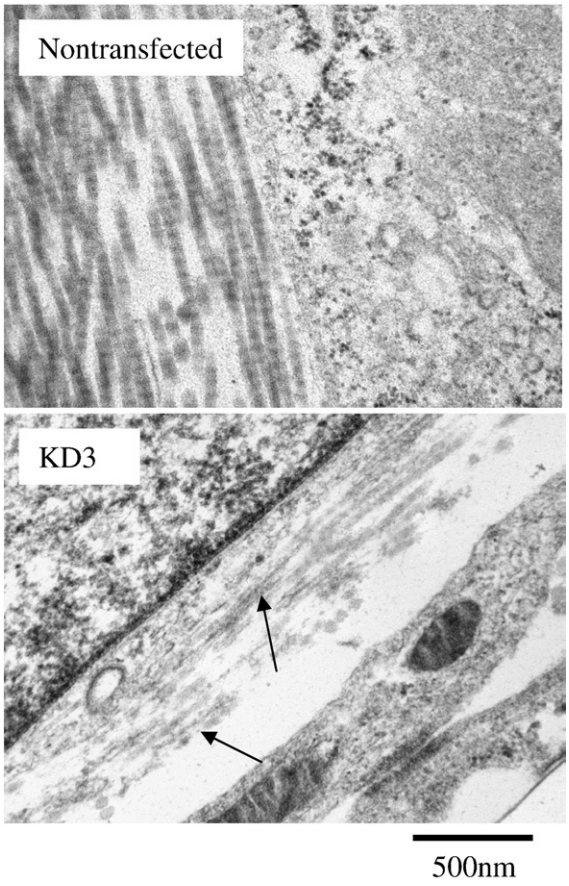

Fig. 3. Over-expression of DDR2/-KD inhibits native banded structure of collagen fibers. Representative electron micrographs show collagen fibers in the ECM. The native nontransfected cells show well-formed collagen fibers with banded structure. In contrast, stable cell lines expressing DDR2/-KD were composed of collagen fibers with weak or no banded structure; arrows indicate fibers with poor banded structure. All samples shown were cultured for two weeks.

of the kinase domain. Additionally, expression of DDR2/-KD did not inhibit the collagen-induced phosphorylation of endogenously expressed DDRs (~125 kDa). Similar results were obtained using MC3T3 cells showing no phosphorylation for the DDR2/-KD protein (data not shown). Figure 1c shows endogenous expression of DDR1 and DDR2 in MC3T3 cells around 125 kDa. Figure 1d shows the expression of DDR2/-KD in three stable cell clones, KD1, KD2, and KD3, which were selected for further studies on the basis of their increasing levels of expression of the DDR2/-KD protein.

Membrane localization of DDR2/-KD was verified using immunocytochemistry followed by confocal microscopy on samples of HEK 293 cells transiently transfected with DDR2/-KD (Fig. 2a). To assess the membrane localization of DDR2/-KD on MC3T3 cells, which are very thinly spread and thus not amenable to confocal microscopy, we used cell-surface biotinylation assays. As shown in Fig. 2b, almost the entire fraction of DDR2/-KD was localized on the cell membrane.

DDR2/-KD alters collagen fiber morphology

To analyze if the expression of DDR2/-KD affects the ultra-structural morphology of collagen fibers assembled in the ECM, we used TEM on samples of native and stable cell lines. As shown in Fig. 3, native cells showed the occurrence of well-formed collagen fibers in the ECM with the characteristic D-periodic banded structure, as early as after one week of culture and for all later time points. In contrast, all

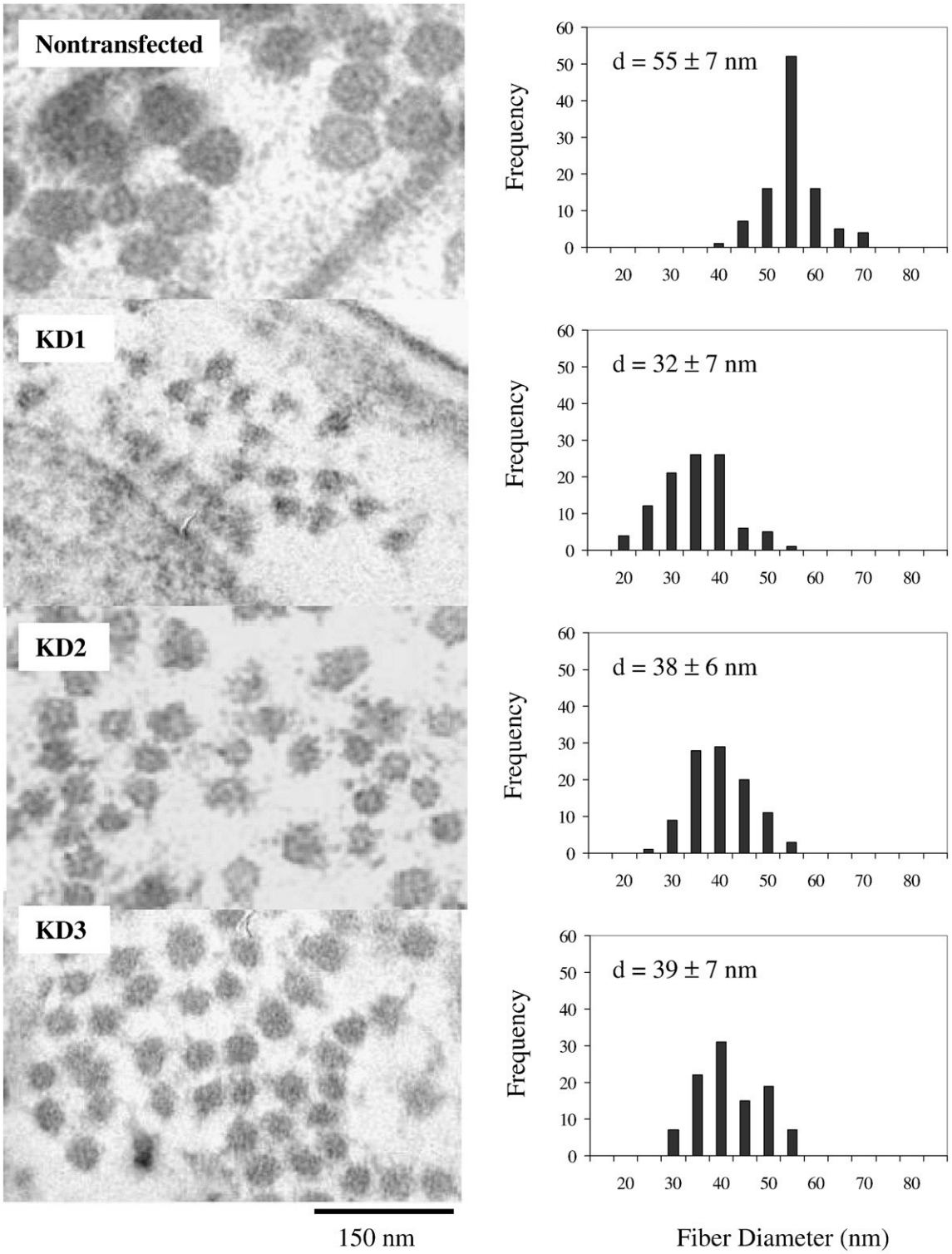

Fig. 4. Over-expression of DDR2/-KD affects collagen fiber diameter. The collagen fiber diameters for all three stable cell lines expressing DDR2/-KD are significantly smaller than nontransfected cells. In the histogram plots, d is the average fiber diameter obtained by measuring 100 fibers for each cell line. The TEM images and measurements shown here were performed on cells cultured for two weeks.

three clones of DDR2/-KD lacked the typical collagen banding pattern for all time points examined (weeks 1, 2 and 3). Fibers with intact native banded structure were observed only occasionally in the DDR2/-KD cell lines.

Next, we asked if expression of DDR2/-KD results in changes in the collagen fiber diameters present in the ECM. Figure 4 shows a frequency distribution of collagen fiber diameter for 100 fibers for each sample at two weeks of culture. A striking reduction in the average fiber diameter was observed for DDR2/-KD expressing cells as compared to native cells. However, no significant change in the average fiber diameter was observed between the different DDR2/-KD clones. To investigate if the expression of DDR2/-KD affects the rate of lateral growth of collagen fibers, we ascertained the average fiber diameter as a function of time for native and the DDR2/-KD clones. As shown in Fig. 5, the native cells exhibited almost no increase in fiber diameter over a period of one to three weeks. The DDR2/-KD samples, in contrast, showed an increase in fiber growth over the first two weeks with little increase in fiber diameter thereafter. The average fiber diameter for all DDR2/-KD samples was significantly lower than that of native cells at each time point. These observations indicate that while the collagen fibers in native cells had reached their steady-state diameter after one week, the collagen fibers in DDR2/-KD had a slower lateral growth rate and approached their steady-state diameters at a later stage.

DDR2/-KD slows the rate of collagen fibrillogenesis

As shown above, our TEM results indicated that the expression of DDR2/-KD exhibited a slower lateral growth rate for collagen endogenously

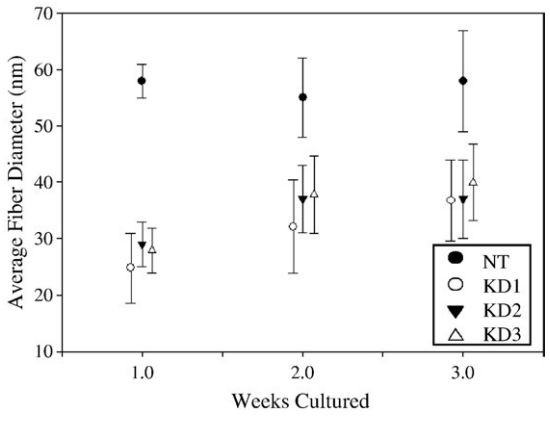

Fig. 5. Over-expression of DDR2/-KD affects collagen fiber diameter growth rate. The lateral growth of collagen fibers in native cells reaches a steady-state value for fiber diameter within one week. Cells expressing DDR2/-KD not only have smaller fiber diameters at each time point, but also continue to grow laterally for one to two weeks of culture. Our results indicate that cells expressing DDR2/-KD reach a steady-state value for lateral growth later than native cells.

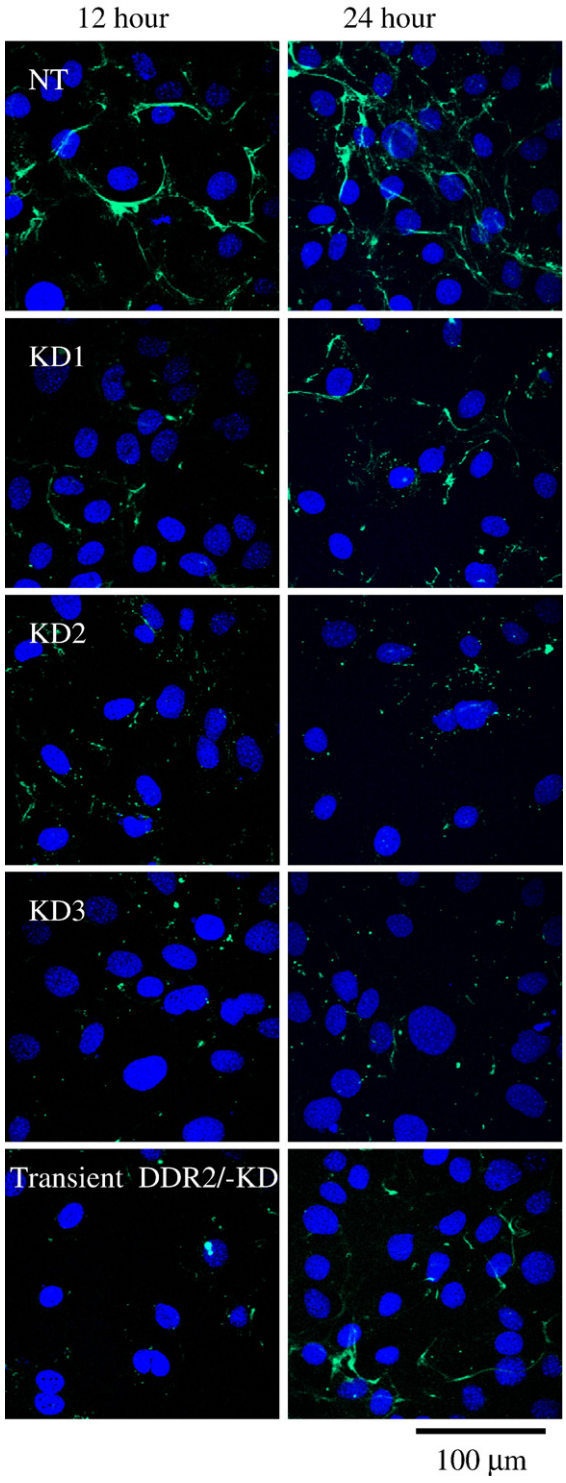

Fig. 6. Collagen fibrillogenesis is reduced in DDR2/-KD-expressing cells. FITC-labeled collagen was added to cell samples and allowed to incubate for the indicated times of 12 h and 24 h. The nontransfected samples show formation and growth of collagen fibers with time. Both stable and transiently transfected cells expressing DDR2/-KD show inhibition of collagen fibrillogenesis. Out of the stable DDR2/-KD cell lines, KD1 shows a relatively higher level of fiber formation, consistent with its lower expression level of DDR2/-KD.

secreted by these cells. To further validate that this inhibition of collagen fibrillogenesis was due to direct interaction of DDR2/-KD with collagen and not due to a delay in secretion of collagen by the stable cell lines, we tested if expression of DDR2/-KD had an effect on the fibrillogenesis of exogenously added collagen. For this purpose, native and transiently or stably transfected DDR2/-KD clones were incubated with fluorescein isothiocyanate (FITC)-labeled monomeric collagen. Figure 6 shows that while the samples with native cells formed collagen fibers rapidly, which increased in length with incubation time, the samples with transiently or stably transfected DDR2/-KD cells showed an inhibition in collagen fibrillogenesis at all time points (12 h and 24 h). Out of the three stably transfected cells, the KD1 clone showed evidence for the formation of slightly more fibers compared to the other two clones, consistent with its lower expression level of DDR2/-KD.

DDR2/-KD leads to reduced collagen deposition

While our TEM micrographs indicate a reduced deposition of collagen in the ECM, the amount of collagen in all samples was quantified by use of the HP assay, a well-established biochemical method for quantification of collagen content.[18,37] To quantify the relative amounts of collagen deposited in the ECM in each sample, native or DDR2/-KD cells were cultured in the presence or in the absence of ascorbic acid and the difference in HP (for the adhered cell and ECM content) between the samples with or without ascorbic acid was ascertained. The amount of soluble collagen present in the conditioned media was not assessed. Further, a cell density assay was used to normalize the cell population due to potential variation in proliferation rates. As shown in Fig. 7, little or no variation occurred in the HP content for all samples when prepared in the absence of ascorbic acid, suggesting that the synthesis of collagen by the cells is not changed in native *versus* DDR2/-KD stable cells. Cell samples prepared in the presence of ascorbic acid exhibited a difference in their HP content of the ECM as a function of DDR2/-KD expression. The normalized HP signal for native cells was significantly larger than the KD1, KD2 (not shown), and KD3 clones, with the HP/collagen content decreasing with increasing expression of DDR2/-KD. The highest expressing clone, KD3, contained only about 45% of the collagen deposited by native cell samples for day 10, and 55% of native collagen deposited for day 14.

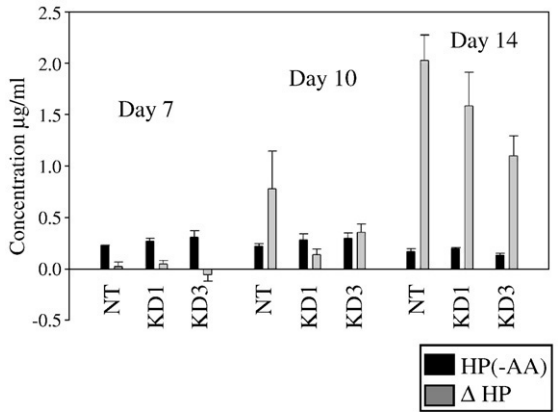

Fig. 7. Quantification of collagen content in the ECM by hydroxyproline (HP) assay reveals reduced deposition of collagen in DDR2/-KD cell lines. In this figure, HP(-AA) is the HP concentration in the adhered cell+ECM portion of the samples made without ascorbic acid. ΔHP is the difference in HP concentration between samples made with and without ascorbic acid. ΔHP is thus the amount of HP that is present as fibrillar collagen in the ECM. In the absence of ascorbic acid, all samples exhibited nearly the same amount of HP. Differences in ΔHP can be clearly observed for nontransfected cells *versus* the cells expressing DDR2/-KD at 10 days and 14 days of culture. Reduced collagen content was observed, consistent with the expression level of DDR2/-KD for stable *versus* nontransfected cells.

Discussion

We demonstrate here that the DDR2 receptor, even when lacking its kinase domain, still remains a regulating factor in collagen fibrillogenesis by cells. Our investigations reveal that the cell surface expression of DDR2/-KD results in collagen fibers in the ECM that are deficient in the native banded structure, have smaller fiber diameter, exhibit delayed kinetics for collagen fibrillogenesis and a reduced collagen deposition in the ECM. These observations are consistent with our earlier results,[18] where we showed that the soluble DDR2 ECD when present in collagen solution *in-vitro*, resulted in a lack of native banded structure, smaller fiber diameters and a delayed kinetics of collagen fibrillogenesis. Our current results thus signify that cell surface-anchored, collagen-binding proteins also have the capacity to regulate collagen fibrillogenesis. We thus elucidate a novel functional role for the expression of DDR2 ECD, which occurs in the full-length DDR2 protein or may be present in other unidentified DDR2 isoforms.

Our results may also provide novel insights into the functional roles of isoforms of DDR1. It is known that DDR1 can exist in five distinct isoforms *in vivo*, DDR1a–e[21], obtained through alternative splicing. Since our DDR2/-KD construct resembles the naturally occurring kinase-dead DDR1 splice variants, DDR1d and DDR1e, and we showed earlier that the soluble DDR1 ECD regulates collagen fibrillogenesis;[19] it is likely that even the DDR1d and DDR1e isoforms along with the full-length DDR1 have a functional role in collagen regulation. Although the splice variants for DDR2 have not been characterized, there is evidence to support their existence. Several protein species for DDR2 have been detected in cultured human smooth muscle cells at various molecular masses: 130 kDa, 90 kDa, 50 kDa and 45 kDa, along with two transcripts at

9.5 kb and 4.5 kb.[10] Independent studies have identified multiple transcripts for DDR2 in cancerous and normal cell lines.[14,22–24] Our results signify the importance of identifying and characterizing the DDR2 isoforms that possess the DDR2 ECD. In addition, our results suggest that other collagen-binding membrane proteins, like integrins and platelet glycoprotein VI, may influence collagen fibrillogenesis, especially if their soluble domains have been demonstrated to regulate collagen fibril formation.[25]

Three DDR2 binding sites have been mapped on the collagen triple helix by us[26] for collagen type 1 and by others using the collagen toolkit for collagen type 2.[27] All of the three reported binding sequences are conserved in the α1 chain of collagen types 1, 2 and 3. The central motif sequence GARGQA-GVMGFO corresponding to amino acids 394–405 has the highest binding affinity for DDR2.[27] This binding site overlaps with the binding site of another soluble collagen-binding protein von Willebrand factor (vWF) in collagen type 3,[27] and is in close proximity to a binding site for decorin on collagen type 1.[28] Although no study has been reported on the effect of vWF on collagen fibrillogenesis, decorin has been found to result in smaller collagen fiber diameter,[2,29] similar to that observed by us for DDR2. It is interesting to note that majority of the collagen-binding proteins known to modulate collagen fibrillogenesis are glycoproteins. Glycosylation of collagen is considered an important factor for binding of both decorin[30] and DDR2[7,8] to collagen. Additionally, collagen glycosylation on the hydroxylysines can have a critical role in regulating fiber diameter in collagen fibrillogenesis.[31,32] Studies addressing the role of glycosylation in DDR2 binding to collagen need further exploration, especially since DDR2 is a glycoprotein with at least one of the binding sites in close proximity to decorin.

Changes in collagen fiber diameter and rate of deposition can also arise due to various types of collagens (heterologous collagen) being incorporated into a fiber. In tissue cultures, Contard et al. showed that fibers with measured diameters of 34 nm or less bound antibodies against collagen type I molecules, while antibodies against collagen type III bound fibers with a diameter between 35 nm and 54 nm.[33] I*n vitro* studies by Birk *et al*. demonstrated that collagen fiber diameter is regulated by collagen type V.[34] They found that pure collagen type V forms small fibers (mean 25 ± 8 nm) with no apparent banded structure seen by TEM. Type V and type I collagens are often known to form mixed fibers with the average collagen diameter increasing with percentage of type I collagen.[34] The collagen fibers assembled in the ECM of mouse osteoblast cells used in this study are composed mostly of collagen types 1, 3 and 5. Further studies are required to determine if DDR2 has varying affinities for these different collagen types and if expression of DDR2 ECD results in differences in collagen composition of the fibers formed. Nevertheless, since our earlier *in-vitro* results with purified collagen type 1 and purified DDR2 ECD also showed the effects of DDR2 on collagen fibrillogenesis, it is unlikely that our observed differences in the present study are largely due to differences in the expression levels of different collagen types.[18]

DDR2 knock-out mice have been shown to possess skeletal defects such as shortening of long bones and irregular growth of flat bones.[35] These defects in knock-out animals have been explained on the basis of impaired chondrocyte and fibroblast proliferation observed in the absence of DDR2. While it is well-known that the ECM can influence cell proliferation, no report exists so far on the ultrastructural collagen morphology for DDR2 knock-out animals. Our results indicate that expression of DDR2 may be critical to regulate collagen deposition, which in turn may affect cell proliferation. A detailed examination of the ECM morphology in DDR2 knock out *versus* wild type animals will provide a more complete understanding of the role of DDR2 in matrix turnover and cell proliferation.

The collagen receptor DDR2 (and likely DDR1) can regulate collagen by two mechanisms: by activating and upregulating MMPs, as reported earlier, and by inhibition of collagen fibrillogenesis as demonstrated in our studies. These two mechanisms give rise to a weakening of the ECM that can influence cell adhesion, migration and proliferation. One may speculate that a weakened ECM would play a different role in developing *versus* adult tissues. In adult tissue, a weakened ECM could result in heightened tumor invasiveness, which is compatible with findings of DDR2 over-expression in malignancies.[9–12] In developing tissue, it is possible that a weaker, or more dynamic, ECM is needed for cell proliferation; such has been reported for the developing heart.[36]

Taken together, our results convey a novel significance of the expression level of DDR2 ECD, found in the full-length DDR2. We conclude that DDR2 ECD expressed on the cell surface can modulate collagen fibrillogenesis. Further, we demonstrate that collagen fibrillogenesis can be regulated by both soluble and cell surface collagen binding proteins in a similar manner.

Materials and Methods

Creation of membrane-anchored, kinase-deficient DDR2 (DDR2/-KD) expression construct

An expression plasmid encoding the kinase-deficient, membrane anchored mouse DDR2 (DDR2/-KD) was generated using the full-length mouse DDR2-myc constructs obtained from Regeneron Pharmaceuticals, Tarrytown, NY.[8] The coding region of the kinase deleted DDR2 (amino acids Met1 through Lys562) was amplified by PCR utilizing the following primers:

forward:5′-3′: AGGATGATCCCGATTCCCAGA
reverse: 5′-3′: CAGTTTCCTGGGGAACTCTTC

and Pfu TURBO polymerase (Stratagene, La Jolla, CA). The resulting PCR product (1689 bp) was subjected to Taq

polymerase to include 3′ A overhangs in the PCR product for enabling ligation immediately into the pcDNA3.1/V5-His-TOPO vector using the Top10 chemically competent cells from Invitrogen. Recombinant clones were identified by restriction analysis using the double digest with KpN1 and EcoRV. The authenticity (i.e., correct orientation and in-frame with the V5 coding region) of the resulting clones were verified by dideoxynucleotide sequencing.

To verify the expression of DDR2/-KD protein, mouse osteoblasts cells, MC3T3-E1 subgroup 4 (from ATCC) were transfected with our DDR2/-KD expression construct using FuGene 6 transfection reagent (Roche, Basel, Switzerland). After 36 h of transfection, the cells were lysed and the lysates subjected to SDS-PAGE followed by Western blotting onto nitrocellulose membranes as described.[18] The membranes were probed with anti-V5 primary antibodies (Invitrogen) (1:1000) and imaged using enhanced chemiluminescence (Amersham Biosciences) after incubation with anti-mouse IgG horseradish peroxidase. The expression of endogenous DDR1 and DDR2 was tested in nontransfected MC3T3 cells using Western blotting with anti DDR1 (sc532, Santa Cruz Biotechnology, Inc) and anti DDR2 (R&D systems, Inc., Minneapolis, MN) antibodies.

To test if DDR2/-KD undergoes collagen-induced tyrosine phosphorylation, HEK293 cells were transiently transfected with DDR2/-KD and serum starved for 12 h after 24 h of transfection. Thereafter the cells were stimulated with 10 μg/ml of collagen type I (Inamed, Fremont, CA) for 90 min. Whole cell lysates were subjected to Western blotting and probed with an anti-phosphotyrosine (4G10) clone (Upstate, Temecula, CA) followed by re-probing with anti-V5 primary antibody (Invitrogen).

Stable cell lines

Mouse osteoblast cells, MC3T3-E1, subgroup-4 (ATCC) were seeded (40–60% confluent) on 100 mm dishes in MEM-α with 10% (v/v) fetal bovine serum and 1% Antibiotic-Antimycotic (pen-G 10,000 units/ml; streptomycin 10,000 μg/ml; amphotericin B 25 μg/ml) from Gibco. MC3T3-E1 cells were subsequently transfected with the DDR2/-KD expression construct using FuGene 6 (Roche Diagnostics). At 36 h after transfection, the cells were incubated with selection medium containing 475 μg/ml of geneticin. After seven days of selection, the individual surviving colonies were transferred to 35 mm dishes and expression of DDR2/-KD in the lysates of the resulting stable cell lines was verified by Western blotting with anti-V5 antibodies. Three stable cell lines designated, KD1, KD2 and KD3, that expressed increasing amounts of DDR2/-KD were utilized in this study.

Immunocytochemistry

HEK293 cells were transiently transfected with DDR2/-KD using Fugene 6 (Roche Diagnostics). After 24 h of transfection, the cells were fixed with 2% (v/v) formalin (Fischer Scientific, Kalamazoo, MI) and the samples were then incubated with a DDR2 antibody raised against its extracellular domain (R&D systems, Inc., Minneapolis, MN). Samples were then incubated with an Alexa Fluor 546 conjugated secondary antibody (Invitrogen, Carlsbad, CA) and imaged on a Zeiss confocal LSM 510 microscope using a 63× oil immersion lens; a 543 nm argon laser was used to excite the fluorochrome. Nontransfected cells were used as controls.

Cell surface biotinylation

MC3T3 cells were cultured as described and transiently transfected with DDR2/-KD construct. At 24 h after transfection, the cells were surface biotinylated by incubation with sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) in PBS for 30 min on ice. The reaction was quenched by washing with 50 mM Tris–HCl, pH 8.0. The cells were lysed in Nonidet P-40 lysis buffer (1% (v/v) Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 2 mM PMSF, 30 μg/ml of aprotinin and 15 μg/ml of leupeptin). The detergent-soluble fraction was recovered by centrifugation for 20 min at 16,000*g*, and the supernatant was subjected to immunoprecipitation (IP) with streptavidin-agarose beads (Pierce) for 2 h to bind the biotinylated proteins. The beads were pelleted by centrifugation and the supernatant was incubated for an additional 2 h with fresh streptavidin-agarose beads to remove any remaining biotinylated proteins. The streptavidin beads from the two IP reactions were pooled together, washed four times in lysis buffer and heated in LDS sample buffer at 75 °F for 10 min to release the bound protein. The supernatant from the second IP reaction was saved and subjected to IP with anti-V5 antibody to collect the intracellular pool of DDR2/-KD. The supernatant from the anti-V5 IP reaction was saved as well and used as the intracellular protein pool. Aliquots of the cell surface (biotinylated) protein pool (IP with strepatavidin), and intracellular DDR2/-KD pool (IP with anti-V5), were subjected to SDS-PAGE and analyzed by Western blotting with anti-V5 antibody. To confirm that the biotinylation protocol did not result in nonspecific labeling of intracellular proteins, aliquots of the biotinylated surface protein pool and intracellular protein pool were analyzed by Western blotting and probed with antibodies against ERK2 kinase (Invitrogen, Carlsbad, CA), an intracellular protein. As an additional control, similar protocols were performed on nonbiotinylated samples, to confirm that that the streptavidin pull-down is specific to biotinylated proteins.

Electron microscopy

Cells were cultured on Thermanox plastic coverslips (Nalge Nunc International, NY) kept in 35 mm dishes, in the presence of 25 μg/ml of ascorbic acid, for one to three weeks as indicated. Samples were fixed in 4% (v/v) glutaraldehyde overnight then kept for 1 h in 1% (w/v) osmium tetroxide, followed by en bloc staining with saturated aqueous uranyl acetate for 1 h. Samples were then dehydrated in a graded ethanol series (30%–100%) and embedded in an epoxy resin. Following polymerization, the 35 mm dishes were discarded to obtain the resin disk. A quick insertion into liquid nitrogen facilitated the removal of the plastic coverslip from the resin disk. The resin disk was cut in half and a drop of liquid resin was used to adhere the two halves such that the cell layers are in juxtaposition. Rectangular portions of resin disk were cut out for sectioning and cell layers were cross-sectioned on a Leica Ultracut UCT ultramicrotome (Leica-Microsystems Wien, Austria). The 80 nm thick sections were picked up on 200-mesh copper grids and post stained with uranyl acetate and Reynolds's lead citrate. Sections were examined in a Zeiss EM 900 TEM (Carl-Zeiss SMT, Peabody, NY) operating at 80 kV. Digital micrographs were captured on an Olympus SIS Megaview III camera (Lakewood, Colorado), at magnifications ranging from 7000 to 85,000×.

Analysis of fiber diameter

Diameters of collagen fibers in the ECM were measured from TEM images using the Image J software (NIH). For each specimen type, at least two identical and independent cell samples were made. At least two TEM grids were made from each sample, and several different regions on each grid were imaged. Collagen fiber diameters were measured on cross-section or longitudinal images observed in TEM micrographs at magnifications of 30,000–50,000×. At least 100 fiber diameters were measured for each specimen type for performing a statistical analysis comprising of average diameter, standard deviation and frequency distribution.

Hydroxyproline (HP) assay

Quantification of HP content in our various cell samples was performed as described.[37] Four cell specimens were analyzed: Native cells and cells stably transfected with DDR2/-KD; namely, colonies KD1, KD2, and KD3. For each specimen, cell samples were prepared in triplicate, with and without ascorbic acid, in order to quantify the amount of fibrillar collagen matrix present in the samples. The analysis of HP content was carried out at days 7, 10 and 14 in cell culture.

Immediately before the HP assay, the cell population in each sample was quantified by a cell proliferation assay using Calcein-Am (BioChemika 17783). The cell samples in six-well plates were washed twice in PBS, after which 2 μl of 1 mM Calcein-AM stock solution was added dropwise to each well and the plates gently swirled to mix the Calcein-AM. Plates were incubated for 30 min at room temperature, after which the fluorescence signal was detected by a fluorescent plate reader using Cytofluor II software (Global Medical Instrumentation, Ramsey, MN) at an excitation wavelength of ~ 485 nm and emission at 530 nm. Once a cell count was estimated for each well, the cell density in each well was normalized with respect to the lowest cell count value measured. The corresponding normalization factor for each sample was thereafter used to normalize the HP measurement for that sample in order to account for any variation in sample growth rate.

After acquiring cell density data, all samples underwent HP analysis as follows. The supernatant was removed by aspiration and contents within the wells were scraped and pipetted into individual 1.5 ml conical O-ring screw-cap tubes (Fisher Scientific 02-681-373). All samples were then brought to a final volume of 50 μl with a final concentration of 4 M sodium hydroxide. Samples were then autoclaved for 20 min at 120 °C, after which 450 μl of chloromine T reagent was added to each sample and incubated at room temperature for 25 min. Thereafter, 500 μl of Ehrlich's reagent was mixed into each sample and incubated at 65°C for 20 min. Finally, the absorbance of each sample was measured at 560 nm using a Beckman DU730 spectrophotometer. The amount of HP in each sample was ascertained by using calibration against a standard curve of HP, obtained using HP ranging from 0.5 μg/ml to10 μg/ml. Collagen content was estimated by considering that hydroxyproline comprises 12.5% of collagen fibers.[37]

Fluorescence microscopy

A fluorescence microscopy-based assay was used to assess the rate of collagen fibrillogenesis as described.[18, 19] Briefly, native cells, or cells transiently transfected with DDR2/-KD or stably transfected DDR2/-KD colonies were cultured on glass coverslips. FITC-labeled collagen type 1 (Sigma Chemicals, MO) was added to each sample at a final concentration of 1 μg/ml. Cells were incubated with collagen for 6 h, 12 h or 24 h. At the end of the incubation period, cells were washed and fixed in 2% (v/v) formalin (Fischer Scientific, Kalamazoo, MI) for 30 min followed by staining with 4′,6-diamidino-2-phenylindole (DAPI). Glass coverslips were then mounted onto microscope slides using ProLong Gold antifade reagent (Invitrogen Molecular Probes P36934) and examined using a 63× objective on a confocal Zeiss LSM 510 microscope. A two-photon laser was used to excite the nuclear stain at 750 nm, and an argon laser at 488 nm was used for the FITC channel. Experiments for each specimen type were repeated at least twice.

Acknowledgements

This work was supported by the NHLBI K25 award 5 K25 HL81442.

References

1. Burgeson, R. E. & Nimni, M. E. (1992). Collagen types. Molecular structure and tissue distribution. *Clin. Orthop. Relat. Res.* **282**.
2. Neame, P. J., Kay, C. J., McQuillan, D. J., Beales, M. P. & Hassell, J. R. (2000). Independent modulation of collagen fibrillogenesis by decorin and lumican. *Cell. Mol. Life Sci.* **57**, 859–863.
3. Halász, K., Kassner, A., Mörgelin, M. & Heinegård, D. (2007). COMP acts as a catalyst in collagen fibrillogenesis. *J. Biol. Chem.* **282**, 31166–31173.
4. Kalamajski, S. & Oldberg, A. (2007). Fibromodulin binds collagen type I via Glu-353 and Lys-355 in leucine-rich repeat 11. *J. Biol. Chem.* **282**, 26740–26745.
5. Rentz, T. J., Poobalarahi, F., Bornstein, P., Sage, E. H. & Bradshaw, A. D. (2007). SPARC regulates processing of procollagen I and collagen fibrillogenesis in dermal fibroblasts. *J. Biol. Chem.* **282**, 22062–22071.
6. Nicolae, C., Ko, Y. P., Miosge, N., Niehoff, A., Studer, D., Enggist, L. *et al.* (2007). Abnormal collagen fibrils in cartilage of matrilin-1/matrilin-3-deficient mice. *J. Biol. Chem.* **282**, 22163–22175.
7. Vogel, W., Gish, G. D., Alves, F. & Pawson, T. (1997). The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol. Cell*, **1**, 13–23.
8. Shrivastava, A., Radziejewski, C., Campbell, E., Kovac, L., McGlynn, M., Ryan, T. E. *et al.* (1997). An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol. Cell*, **1**, 25–34.
9. Yamanaka, R., Arao, T., Yajima, N., Tsuchiya, N., Homma, J., Tanaka, R. *et al.* (2006). Identification of expressed genes characterizing long-term survival in malignant glioma patients. *Oncogene*, **25**, 5994–6002.
10. Johansson, F. K., Goransson, H. & Westermark, B. (2005). Expression analysis of genes involved in brain tumor progression driven by retroviral insertional mutagenesis in mice. *Oncogene*, **24**, 3896–3905.
11. Ford, C. E., Lau, S. K., Zhu, C. Q., Andersson, T., Tsao, M. S. & Vogel, W. F. (2007). Expression and mutation

analysis of the discoidin domain receptors 1 and 2 in non-small cell lung carcinoma. *Br. J. Cancer*, **96**, 808–814.
12. Chua, H. H., Yeh, T. H., Wang, Y. P., Huang, Y. T., Sheen, T. S., Lo, Y. C. *et al.* (2008). Upregulation of discoidin domain receptor 2 in nasopharyngeal carcinoma. *Head Neck*, **30**, 427–436.
13. Franco, C., Hou, G. & Bendeck, M. (2002). Collagens, integrins, and the discoidin domain receptors in arterial occlusive disease. *Trends Cardiovasc. Med.* **12**, 143–148.
14. Ferri, N., Carragher, N. O. & Raines, E. W. (2004). Role of Discoidin domain receptors 1 and 2 in human smooth muscle cell-mediated collagen remodeling: potential implications in atherosclerosis and lymphangioleiomyomatosis. *Am. J. Pathol.* **164**, 1575–1585.
15. Li, W., Zhang, Y. Q., Liu, X. P., Yao, L. B. & Sun, L. (2005). Regular expression of discoidin domain receptor 2 in the improved adjuvant-induced animal model for rheumatoid arthritis. *Clin. Med. Sci. J.* **20**, 133–137.
16. Xu, L., Peng, H., Wu, D., Hu, K., Goldring, M. B., Olsen, B. R. & Li, Y. (2005). Activation of the discoidin domain receptor 2 induces expression of matrix metalloproteinase 13 associated with osteoarthritis in mice. *J. Biol. Chem.* **280**, 548–555.
17. Vogel, W. (1999). Discoidin domain receptors: structural relations and functional implications. *FASEB J.* **13**, S77–S82.
18. Mihai, C., Iscru, D. F., Druhan, L. J., Elton, T. S. & Agarwal, G. (2006). Discoidin domain receptor 2 inhibits fibrillogenesis of collagen type I. *J. Mol. Biol.* **361**, 864–876.
19. Agarwal, G., Mihai, C. & Iscru, D. F. (2007). Interaction of discoidin domain receptor 1 with collagen type 1. *J. Mol. Biol.* **367**, 443–455.
20. Pornprasertsuk, S., Duarte, W. R., Mochida, Y. & Yamauchi, M. (2005). Overexpression of lysyl hydroxylase-2b leads to defective collagen fibrillogenesis and matrix mineralization. *J. Bone Miner. Res.* **20**, 81–87.
21. Alves, F., Saupe, S., Ledwon, M., Schaub, F., Hiddemann, W. & Vogel, W. F. (2001). Identification of two novel, kinase-deficient variants of discoidin domain receptor 1: differential expression in human colon cancer cell lines. *FASEB J.* **15**, 1321–1323.
22. Alves, F., Vogel, W., Mossie, K., Millauer, B., Hofler, H. & Ullrich, A. (1995). Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. *Oncogene*, **10**, 609–618.
23. Karn, T., Holtrich, U., Brauninger, A., Bohme, B., Wolf, G., Rubsamen-Waigmann, H. & Strebhardt, K. (1993). Structure, expression and chromosomal mapping of TKT from man and mouse: a new subclass of receptor tyrosine kinases with a factor VIII-like domain. *Oncogene*, **8**, 3433–3440.
24. Lai, C. & Lemke, G. (1994). Structure and expression of the Tyro 10 receptor tyrosine kinase. *Oncogene*, **9**, 877–883.
25. Jokinen, J., Dadu, E., Nykvist, P., Käpylä, J., White, D. J., Ivaska, J. *et al.* (2004). Integrin-mediated cell adhesion to type I collagen fibrils. *J. Biol. Chem.* **279**, 31956–31963.
26. Agarwal, G., Kovac, L., Radziejewski, C. & Samuelsson, S. (2002). Binding of discoidin domain receptor 2 to collagen I: an atomic force microscopy investigation. *Biochemistry*, **41**, 11091–11098.
27. Konitsiotis, A. D., Raynal, N., Bihan, D., Hohenester, E., Farndale, R. W. & Leitinger, B. (2008). Characterization of high affinity binding motifs for the discoidin domain recptor DDR2 in collagen. *J. Biol. Chem.* **283**, 6861–6868.
28. Di Lullo, G. A., Sweeney, S. M., Korkko, J., Ala-Kokko, L. & San Antonio, J. D. (2002). Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *J. Biol. Chem.* **277**, 4223–4231.
29. Rada, J. A., Cornuet, P. K. & Hassell, J. R. (1993). Regulation of corneal collagen fibrillogenesis in vitro by corneal proteoglycan (lumican and decorin) core proteins. *Exp. Eye Res.* **56**, 635–648.
30. Rühland, C., Schönherr, E., Robenek, H., Hansen, U., Iozzo, R. V., Bruckner, P. & Seidler, D. G. (2007). The glycosaminoglycan chain of decorin plays an important role in collagen fibril formation at the early stages of fibrillogenesis. *FEBS J.* **274**, 4246–4255.
31. Notbohm, H., Nokelainen, M., Myllyharju, J., Fietzek, P. P., Müller, P. K. & Kivirikko, K. I. (1999). Recombinant human type II collagens with low and high levels of hydroxylysine and its glycosylated forms show marked differences in fibrillgenesis in vitro. *J. Biol. Chem.* **274**, 8988–8992.
32. Reigle, K. L., Di Lullo, G., Turner, K. R., Last, J. A., Chervoneva, I., Birk, D. E. *et al.* (2008). Non-enzymatic glycation of type I collagen diminishes collagen-proteoglycan binding and weakens cell adhesion. *J. Cell Biochem.* **104**, 1684–1698.
33. Contard, P., Lloydstone, J., Perlish, J. S. & Fleischmajer, R. (1993). Collagen fibrillogenesis in a three-dimensional fibroblast cell culture system. *Cell Tissue Res.* **273**, 571–575.
34. Birk, D. E., Fitch, J. M., Babiarz, J. M., Doane, K. J. & Linesenmayer, T. F. (1990). Collagen Fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. *J. Cell Sci.* **95**, 649–657.
35. Labrador, J. P., Azcoitia, V., Tuckermann, J., Lin, C., Olaso, E., Mañes, S. *et al.* (2001). The collagen receptor DDR2 regulates proliferation and its elimination leads to dwarfism. *EMBO Rep.* **2**, 446–452.
36. Morales, M. O., Price, R. L. & Goldsmith, E. C. (2005). Expression of discoidin domain receptor 2 (DDR2) in the developing heart. *Microsc. Microanal.* **11**, 260–267.
37. Reddy, G. K. & Enwemeka, C. S. (1996). A simplified method for the analysis of hydroxyproline in biological tissues. *Clin. Biochem.* **29**, 225–229.