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Shedding of NG2 by MMP-13 Attenuates Anoikis

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Disruption of cell–matrix interactions can lead to anoikis—apoptosis due to loss of matrix contacts. We previously showed that Nerve/glial antigen 2 (NG2) is a novel anoikis receptor. Specifically, overexpression of *NG2* leads to anoikis propagation, whereas its suppression leads to anoikis attenuation. Interestingly, NG2 expression decreases in late anoikis, suggesting that NG2 reduction is also critical to this process. Thus, we hypothesized that NG2 undergoes cleavage to curtail anoikis propagation. Further, since matrix metalloproteinases (MMPs) cleave cell surface receptors, play a major role in modulating apoptosis, and are associated with death receptor cleavage during apoptosis, we further hypothesized that cleavage of NG2 could be mediated by MMPs to regulate anoikis. Indeed, anoikis conditions triggered release of the NG2 extracellular domain into condition media during late apoptosis, and this coincided with increased MMP-13 expression. Treatment with an MMP-13 inhibitor and MMP-13 siRNA increased anoikis, since these treatments blocked NG2 release. Further, NG2-positive cells exhibited increased anoikis upon MMP-13 inhibition, whereas MMP-13 inhibition did not increase anoikis in NG2-null cells, corroborating that retention of NG2 on the cell membrane is critical for sustaining anoikis, and its cleavage for mediating anoikis attenuation. Similarly, *NG2* suppression with siRNA inhibited NG2 release and anoikis. In contrast, *MMP-13* overexpression or exogenous MMP-13 reduced anoikis by more effectively shedding NG2. In conclusion, maintenance of NG2 on the cell surface promotes anoikis propagation, whereas its shedding by MMP-13 actions attenuates anoikis. Given that these findings are derived in the context of periodontal ligament fibroblasts, these data have implications for periodontal inflammation and periodontal disease pathogenesis.

Introduction

Apoptosis or programmed cell death is a highly regulated cellular process whose characteristic features include cellular shrinkage, nuclear condensation, and chromosomal DNA fragmentation. Apoptosis is central to many cell and tissue processes and disease mechanisms, including normal embryonic development and inflammation. Excessive apoptosis leads to atrophy as in neurodegenerative diseases, whereas insufficient apoptosis contributes to cancer processes. Anoikis is a form of programmed cell death mediated by loss of extracellular matrix (ECM) contacts. The mechanisms that regulate anoikis are not fully understood. We recently identified a novel anoikis receptor, the Nerve/glial antigen 2 (NG2) proteoglycan, yet the mechanism by which it regulates anoikis propagation has not been determined. The current investigation examines this process.

NG2 is a transmembrane proteoglycan receptor that interacts with ECM molecules, including type VI collagen, and with other cell surface components, including beta-1 integrins, to mediate cell adhesion and proliferation (Burg *et al.*, 1997; Tillet *et al.*, 1997; Goretzki *et al.*, 1999; Fukushi *et al.*, 2004; Makagiansar *et al.*, 2004; Makagiansar *et al.*, 2007; Chekenya *et al.*, 2008; Stallcup and Huang, 2008; Cattaruzza *et al.*, 2013). The cytoplasmic domain of NG2 interacts with scaffolding proteins, such as MUPP1 and GRIP1, and with kinases, such as PKCα and ERK (Barritt *et al.*, 2000; Stegmüller *et al.*, 2003; Makagiansar *et al.*, 2004; Makagiansar *et al.*, 2007). We previously reported that anoikis signals transmitted by increased NG2 levels lead to decreases in PKCα and pFAK levels (Joo *et al.*, 2008). Overexpression of *NG2* decreases both PKCα levels and phosphorylation of FAK, while suppression of *PKCα* decreases FAK phosphorylation, indicating that NG2 regulates FAK phosphorylation through PKCα in fibroblasts. In addition, since PKCα can phosphorylate NG2 and change its surface distribution (Makagiansar *et al.*, 2004), changes in PKCα levels could impact cell surface localization and function of NG2.

We previously found that NG2 expression decreased in late anoikis, suggesting that this NG2 reduction was also

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critical to the anoikis process. This reduction in NG2 surface expression was accompanied by increased NG2 levels in the extracellular media, suggesting that proteolytic processing of NG2 was part of anoikis propagation. This became the focus of the current study.

Many cell lines that express the full-length 300-kDa NG2 core protein—the mature and transmembranal form—also release two truncated forms into the medium (Nishiyama *et al.*, 1995). One truncated 290-kDa form lacks the cytoplasmic domain but contains almost the entire ectodomain. The other truncated form is a 275-kDa species that lacks the cytoplasmic domain and at least 64 amino acids of the ectodomain. Mild trypsinization of B49 cells generates the 275-kDa species, suggesting that this component is produced by proteolysis of the 300-kDa form. The intact 300-kDa form and a truncated 275-kDa form are expressed at the surface of an *NG2*-transfected cell line U251NG52. Conversion of the 300-kDa species to the 275-kDa form in U251NG52 cells is stimulated by reagents such as phorbol esters, which activate protein kinase C.

Phorbol esters are also known to induce expression of matrix metalloproteinases (MMPs), such as collagenase and stromelysin. Thus, phorbol-ester-mediated activation of MMPs could be responsible for cleavage of the 300-kDa NG2 core protein. Findings from other studies have suggested that certain MMPs, including MMP-9, MT1-MMP, ADAM8, and ADAM9, may be involved in NG2 processing (Larsen *et al.*, 2003; Asher *et al.*, 2005). It is unclear whether a specific MMP is associated with shedding of NG2 from the cell surface that thereby contributes to anoikis regulation.

MMPs, zinc-dependent endopeptidases that degrade ECM proteins, are associated with modulating different cell behaviors, including cell proliferation, migration, and angiogenesis. Further evidence also indicates that MMPs regulate apoptosis. Specifically, MMPs and their inhibitors (TIMPs) are thought to play an important role in regulating cleavage of death and growth factor receptors in apoptosis regulation (Ahonen *et al.*, 2003; Tran *et al.*, 2010). Similarly, we hypothesized that proteolytic cleavage of membrane-bound NG2 by MMPs may be involved in anoikis regulation. Indeed, our findings show for the first time that cleavage of an ECM receptor, NG2, by an MMP-mediated process regulates anoikis. Although there are a few reports of MMPs regulating apoptosis via cleavage of death and growth factor receptors, to our knowledge, this is the first report of cleavage or shedding of an ECM proteoglycan receptor by MMPs in the regulation of anoikis. Given that these findings are derived in the context of periodontal ligament fibroblasts, these data have implications for periodontal inflammation and periodontal disease pathogenesis.

Materials and Methods

Cell culture

Primary human periodontal ligament fibroblasts were obtained as described (Kapila *et al.*, 1999). Fibroblasts were maintained in alpha-minimal essential medium containing 10% fetal bovine serum and 1% penicillin/streptomycin/fungizone. NG2-null mouse aortic smooth muscle cells and wild-type control aortic smooth muscle cells (Grako *et al.*, 1999) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin–streptomycin. Conditioned media (CM) consisted of regular cell culture media as described earlier for each cell type but obtained from cells after treatment with the fibronectin V+H− (anoikis) or V+H+ (control) proteins and assayed in different experiments.

Fibronectin proteins and anoikis

Recombinant fibronectin proteins containing an alternatively spliced V region (V+) and either an unmutated (H+) [V+H+] or a mutated nonfunctional, high-affinity, heparin-binding domain (H−) [V+H−] were purified as described (Kapila *et al.*, 1999) and used to treat cells to trigger anoikis as previously described (Joo *et al.*, 2008). Anoikis or control conditions were mediated using 40 μg/mL of fibronectin proteins, V+H− or V+H+, respectively.

Apoptotic cell death detection by ELISA in primary human periodontal ligament fibroblast cells

DNA fragmentation in cell lysates and supernatants was assayed after incubation under anoikis or control conditions using the cell-death-detection enzyme-linked immunosorbent assay (ELISA) plus kit (Roche Molecular Biochemicals). The ELISA, which measures cytoplasmic histone-associated DNA fragments after induced cell death, was performed according to the manufacturer's instructions. Colorimetric detection and quantification of the ELISA were performed on a microplate reader (Molecular Devices).

Immunoprecipitation in primary human periodontal ligament fibroblast cells and NG2-null mouse aortic smooth muscle cells

Levels of NG2 proteoglycan were assessed by standard immunoprecipitation. Cell lysates and CM were pretreated with 0.2 U of chondroitinase ABC for 1 h at 37°C and then incubated with 50 µL of protein A-Sepharose beads (Amersham Pharmacia Biotech) for 2 h. Supernatants were incubated with 2 µL of B5 mouse monoclonal antibody overnight. Samples were then pelleted, washed three times with phosphate-buffered saline (PBS) buffer, pelleted again, and eluted from the beads by addition of sample buffer and boiling for 5 min. Samples were electrophoretically resolved by standard methods on 4–12% gradient polyacrylamide gels (Invitrogen). After electrophoresis, gels were transferred to nitrocellulose by standard methods. The immunoblots were probed with a primary antibody, anti-NG2 rabbit polyclonal antibody (Chemicon), and then incubated with a secondary anti-rabbit antibody conjugated to peroxidase (Santa Cruz Biotechnology). Bound antibody was detected using the ECL-plus detection system (Pierce).

Western blotting

Samples for NG2 detection from the cellular components were lysed using standard RIPA buffer and then pretreated with 0.2 U of chondroitinase ABC for 1 h at 37°C prior to analysis. Samples for NG2 detection from the CM were collected and concentrated prior to analysis. Samples were normalized by equal volume for CM analysis or equal protein for cell lysate analysis and then electrophoretically

resolved using standard SDS-PAGE and western blotting. Truncated forms of NG2 were detected with the 1657 and 1466 polyclonal antibodies (Nishiyama *et al.*, 1991, 1995). Intact NG2 was detected with the B5 monoclonal antibody (Lim *et al.*, 2007) and an NG2 polyclonal antibody (Chemicon). Polyclonal MMP-2, MMP-9, and MMP-13 antibodies were from Santa Cruz Biotechnology (Santa Cruz Biotech).

RNAi

Primary human periodontal ligament fibroblast cells were transiently transfected with *MMP-13* siRNA, *NG2* siRNA, or Stealth RNAi Negative Control (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen). Cells were then treated under anoikis or control conditions in serum-free medium for experiments. To monitor gene silencing, cell extracts were assessed by western blotting 24, 36, and 48 h after transfection.

MMP inhibition

To explore whether MMP-13 was involved in the proteolysis of the NG2 proteoglycan, cells were pretreated with 1 to 15 nM of an MMP-13 inhibitor (CAS 544678-85-5; Calbiochem) for 2 h and then treated with the FN protein overnight. NG2 levels in cell lysates and CM were then assessed using standard immunoprecipitation methods with antibodies described in the ''Western blotting section'' heading. The MMP-13-specific inhibitor used in these studies potently inhibits MMP-13 activity (IC50 = 8 nM) with expected selectivity over MMP-1, -2, -3, -7, -8, -9, -10, -12, -14, and -16 as determined by conformational structure analysis. It has been shown to bind to the MMP-13 catalytic domain and act as a nonzinc-chelating inhibitor.

DNA transfection in primary human periodontal ligament fibroblast cells

At 60–80% confluency, cells in six-well tissue culture plates were transiently transfected with *MMP-13* cDNA or vector control using Lipofectamine 2000 (Invitrogen) for 6 h, washed, and incubated with αMEM/10%FBS for 36 h. Cells were then treated under anoikis or control conditions in serum-free medium for experiments. Transfection efficiency was assessed by western blot of cell extracts 24, 36, and 48 h after transfection.

Exogenous MMP-13

Recombinant human MMP-13 (Sigma) was activated with 2 mM APMA (*p*-aminophenylmercuric acetate) and incubated at 37°C for 30 min according to the manufacturer's recommendations, and then dialyzed against PBS using a membrane with a nominal molecular weight limit of 10,000 Da (Millipore). Primary human ligament fibroblast cells were pretreated with 100 ng/mL of activated exogenous MMP-13 for 2 h and then treated under anoikis or control conditions overnight. NG2 levels in cell lysates and CM were assessed by immunoprecipitation.

Statistical analysis

Data were analyzed by a two-way analysis of variance and the intergroup differences were determined by Fisher's PLSD test. Intergroup differences were analyzed by an unpaired Student's *t*-test. $p < 0.05$ was considered significant. Values are given as mean ± SEM. All experiments were performed in triplicate.

Results

Anoikis activates MMP-13 expression and NG2 cleavage

Previously, NG2 was identified as a novel anoikis receptor, such that its upregulation led to anoikis and its suppression rescued cells from anoikis (Joo *et al.*, 2008). Interestingly, this report also demonstrated that under anoikis conditions, NG2 expression levels initially increased and then decreased in cell lysates over time. However, overexpression experiments confirmed that it was the increased levels of NG2 that were driving anoikis. So, what was the significance of the decreasing levels of NG2 in the cell lysates over time during anoikis, when it was clear that the high NG2 expression levels were triggering anoikis? To answer this question, we hypothesized that NG2 might be undergoing cell surface cleavage as a means of controlling its actions during anoikis. To test this possibility, we examined whether an NG2 cleavage product was being released into the CM of cells undergoing anoikis. Indeed, western blot analysis of cell CM assayed after 24 h revealed the presence of an NG2 component in the media of cells undergoing anoikis, but not in those under control survival conditions (Fig. 1A). In fact, under anoikis conditions, an NG2 component appeared at increasing amounts in the CM while simultaneously disappearing from the cell surface lysates in a time-dependent manner (Fig. 1B-a). Given this finding, we reasoned that NG2 was undergoing proteolytic cleavage at the cell surface by an MMP. Of the MMPs that are expressed at basal levels by these periodontal ligament fibroblasts or can be induced in the context of periodontal inflammation (Kapila *et al.*, 1996; Hernandez *et al.*, 2006, 2007; Silva *et al.*, 2008; Hernández Ríos *et al.*, 2009; Paula-Silva *et al.*, 2009; Miao *et al.*, 2011, 2014; Ahn *et al.*, 2014; Leppilahti *et al.*, 2014), MMP-13 was the only protease that exhibited increased expression levels under anoikis conditions (Fig. 1B-b). Therefore, MMP-13 was examined over time, and its expression also increased time dependently like the NG2 component in the media of cells undergoing anoikis (Fig. 1B-a). However, initial increase of MMP-13 preceded the appearance of the NG2 component in the media, which is consistent with the idea that MMP-13 undergoes increased expression and subsequent activation by a pro-anoikis ECM, and increase and activation of MMP-13 subsequently triggers the cleavage of NG2, thereby mediating the release or shedding of NG2 into the media.

As a further confirmation that NG2 was undergoing proteolytic cleavage and shedding under anoikis conditions, the NG2 from the CM was examined with antibodies that specifically detect the ectodomain and cytodomain of NG2. Western blotting experiments confirmed that the form of NG2 that appeared in the media contained the ectodomain, which is accessible to cell surface cleavage, but not the cytodomain, which is intracellular and not accessible to extracellular cleavage (Fig. 1B-c).

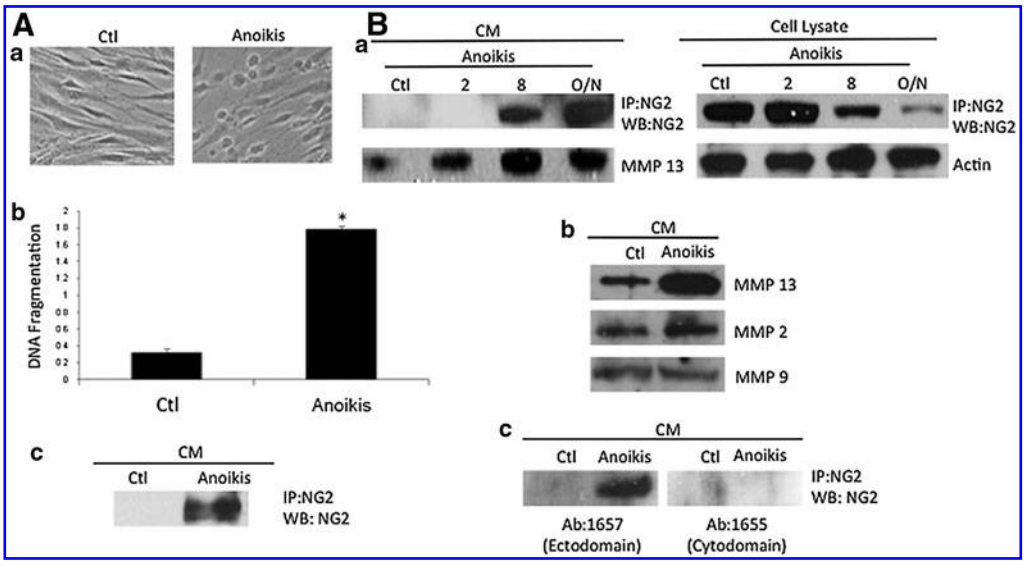

FIG. 1. Anoikis conditions trigger cleavage and release of an NG2 cleavage product and increases in MMP-13 expression. **(A) (a)** Primary human periodontal ligament fibroblast cell morphology and **(b)** cell death ELISA assessed in cells treated under anoikis or control (Ctl) conditions (fibronectin proteins V+H− or V+H+, respectively; 40 μg/mL) for 18 h. **(c)** Imunoprecipitation and western blotting of NG2 in conditioned media (CM) assessed under the same conditions as on the top. **(B) (a)** Imunoprecipitation and western blotting of NG2 plus western blotting for MMP-13 in primary human periodontal ligament fibroblast cell lysates and CM under the same conditions as in **(A)** and sampled at 2, 8, and 18 h (overnight, O/N). Actin served as a loading control. **(b)** Western blotting for MMP-13, MMP-2, and MMP-9 was assessed in CM from samples treated under the same conditions as in **(A)**. **(c)** Imunoprecipitation and western blotting of NG2 under anoikis conditions with specific ectodomain and cytodomain antibodies to NG2. Values are mean ± SEM. $*p < 0.05$ versus control. ELISA, enzyme-linked immunosorbent assay; MMP, matrix metalloproteinase; NG2, Nerve/glial antigen 2.

To once again validate the critical role of NG2 and its shedding in anoikis regulation, *NG2* was silenced to examine its effects on anoikis mediation. As expected, silencing *NG2* with siRNA led to decreased levels of NG2 in CM and a reduced level of DNA fragmentation (Fig. 2A, B).

Inhibiting MMP-13 activity and expression decreases NG2 cleavage and thereby increases DNA fragmentation

To examine whether the functional activity of MMP-13 was critical to NG2 shedding during anoikis regulation, a chemical inhibitor of MMP-13 was examined in this context. Chemical inhibition of MMP-13 activity led to a progressive drop in NG2 levels in the media, and a progressive increase in DNA fragmentation upon treatment with increasing doses of an MMP-13 inhibitor under anoikis conditions (Fig. 3A, B). Thus, MMP-13 activity is required for NG2 shedding and for limiting anoikis.

To directly examine the critical role of MMP-13 in regulating NG2 cleavage and shedding during anoikis propagation, *MMP-13* expression was silenced using siRNA and examined in this mechanism. Silencing of *MMP-13* confirmed that MMP-13 is critical to mediating the cleavage and shedding of NG2 under anoikis conditions, since NG2 levels dropped significantly in the CM when reduced/silenced levels of *MMP-13* could no longer mediate NG2 shedding (Fig. 3C a and b). Importantly, silencing *MMP-13* and inhibiting NG2 shedding led to increased levels of DNA fragmentation under anoikis conditions (Fig. 3D). These data corroborate that the maintenance of high levels of uncleaved NG2 on the cell surface is an important factor for

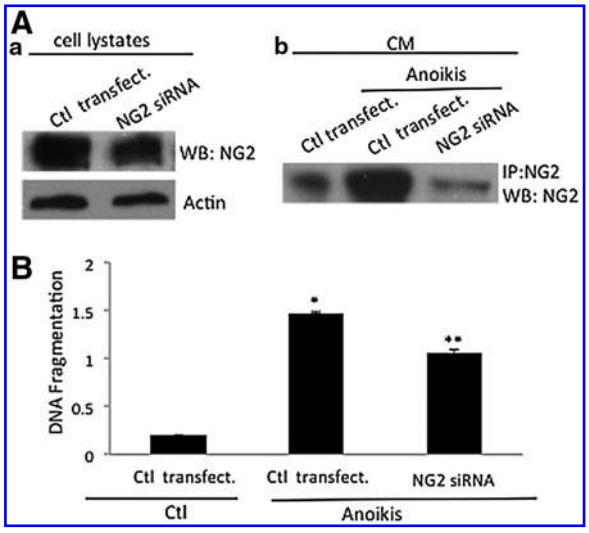

FIG. 2. Silencing *NG2* with siRNA led to decreased levels of NG2 in CM and a reduced level of DNA fragmentation under anoikis conditions. **(A)** Western blotting of primary human periodontal ligament fibroblast cells transfected with **(a)** *NG2* siRNA or control siRNA (100 pmol). Actin served as a loading control. **(b)** Imunoprecipitation and western blotting for NG2 from CM from cells treated under anoikis conditions (fibronectin V+H−; 40 μg/mL). **(B)** Cell death ELISA for DNA fragmentation assessed under anoikis conditions for *NG2*-transfected, control–transfected, and control-transfected primary human periodontal ligament fibroblast cells under control conditions (fibronectin V+H+; 40 μg). Values are mean ± SEM. $*p < 0.05$ versus control. $**p < 0.05$ versus anoikis control.

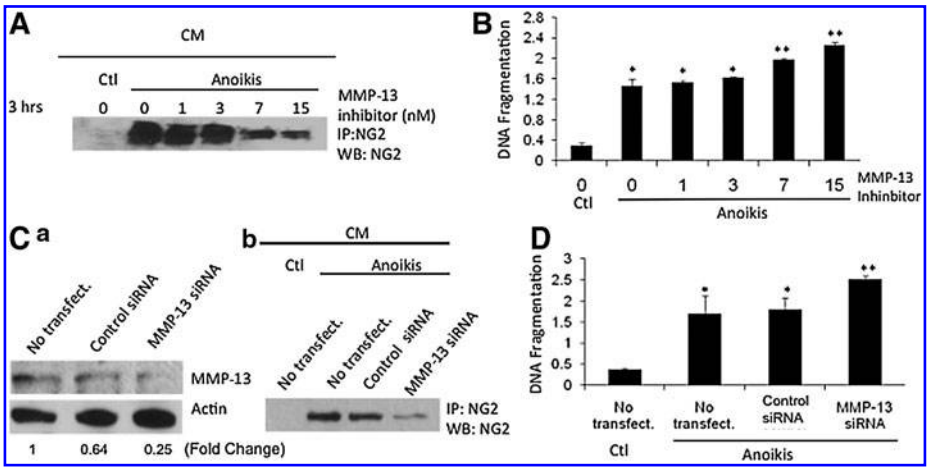

FIG. 3. An MMP-13 inhibitor and *MMP-13* siRNA trigger increased DNA fragmentation due to reduced shedding of NG2. **(A)** Imunoprecipitation and western blotting of NG2 in primary human periodontal ligament fibroblast cells pretreated with exogenous 1 to 15 nM of an MMP-13-specific chemical inhibitor and then treated under anoikis (fibronectin V+H−; 40 μg/mL) or control (Ctl; fibronectin V+H+; 40 μg/mL) conditions for 18 h. **(B)** Cell death ELISA assessed under the same conditions as in **(A)**. **(C)** Western blotting of MMP-13 **(a)** and imunoprecipitation and western blotting of NG2 **(b)** for primary human periodontal ligament fibroblast cells transfected with *MMP-13* siRNA or control transfected (200 pmol). After transfection, cells were treated under anoikis (fibronectin protein V+H−; 40 μg/mL) or control (fibronectin V+H+; 40 μg/mL) conditions for 18 h. Actin was used as a loading control. Fold suppression in MMP-13 expression relative to the actin control is indicated below the blots. **(D)** Cell death ELISA assessed under the same conditions as in **(C)**. Values are mean ± SEM. $*p < 0.05$ versus anoikis control. $**p < 0.05$ versus control.

promoting anoikis propagation, since blocking its cleavage and release triggered higher levels of DNA fragmentation under anoikis conditions.

MMP-13 inhibition does not alter anoikis in NG2-null cells

To further confirm that cellular retention of uncleaved NG2 is required for anoikis propagation, NG2-null ($NG2^{-/-}$) and NG2-positive ($NG2^{+/+}$) cells were pretreated with an MMP-13 inhibitor and then compared under anoikis conditions. These data showed that NG2-positive cells treated with an MMP-13 inhibitor exhibited lower levels of NG2 shedding into the CM, higher levels of NG2 cellular retention, and higher levels of DNA fragmentation under anoikis conditions compared with controls (Fig. 4A-a and b, B-a and b). In contrast, those not treated with an MMP-13 inhibitor showed higher levels of NG2 shedding into the CM, lower levels of NG2 cellular retention, and lower levels of DNA fragmentation under anoikis conditions. It is noteworthy that the DNA fragmentation levels are very different in these two cell types under anoikis conditions and compared with controls because of their NG2-null and NG2-positive status. NG2-null cells, which do not express NG2, exhibited lower levels of DNA fragmentation compared with the NG2 wild-type cells under anoikis conditions. Thus, like in our previous study, NG2-null cells were resistant to anoikis (Joo *et al.*, 2008). The NG2-null cells also exhibited comparable levels of DNA fragmentation regardless of whether or not they were treated with an MMP-13 inhibitor under anoikis conditions. Parenthetically, the MMP-13 inhibitor limits MMP-13 enzymatic activity but not MMP-13 expression levels (Fig. 4A-a). Taken in aggregate, these data confirm that inhibiting cleavage of NG2 by inhibiting MMP-13 activity leads to retention of NG2 on the cell, which promotes DNA fragmentation under anoikis conditions.

Overexpression of MMP-13 inhibits anoikis

To further examine the contribution of MMP-13 to NG2 cleavage and anoikis regulation, *MMP-13* was overexpressed in primary human periodontal ligament fibroblast cells and its effects were examined in the context of anoikis. Overexpression of *MMP-13* led to increased levels of NG2 release into the CM, and decreased levels of DNA fragmentation under anoikis conditions (Fig. 5A, B-a and b). Controls exhibited lower levels of NG2 release into the media, and higher levels of DNA fragmentation.

Addition of exogenous MMP-13 to cells also led to increased NG2 shedding into the CM and decreased levels of DNA fragmentation under anoikis conditions compared with controls (Fig. 5C, D).

Discussion

In this study, we demonstrate that MMP-13 is involved in the shedding of NG2 to attenuate anoikis propagation in periodontal ligament fibroblasts. Our data support the concept that MMP-13 undergoes both upregulation and subsequent activation in CM upon anoikis to mediate this process, since MMP-13 protein expression increases over time (Fig. 1B) and inhibition of its activity attenuates this process (Fig. 3A, B). Extracellular or pericellular activation is a common mechanism by which MMPs undergo activation. MMP-13 can undergo extracellular activation by urokinase plasminogen activator, an enzyme that is expressed by primary periodontal ligament cells and activated by fibronectin fragments in these cells (Kapila *et al.*, 1996; Zijlstra *et al.*, 2004). A likely mechanism driving the upregulation of MMP-13 may stem from changes in intracellular signaling mediated via NG2 or fibronectin fragments upon anoikis propagation. We have shown that NG2 alters PKC and FAK survival signaling pathways to mediate anoikis induced by

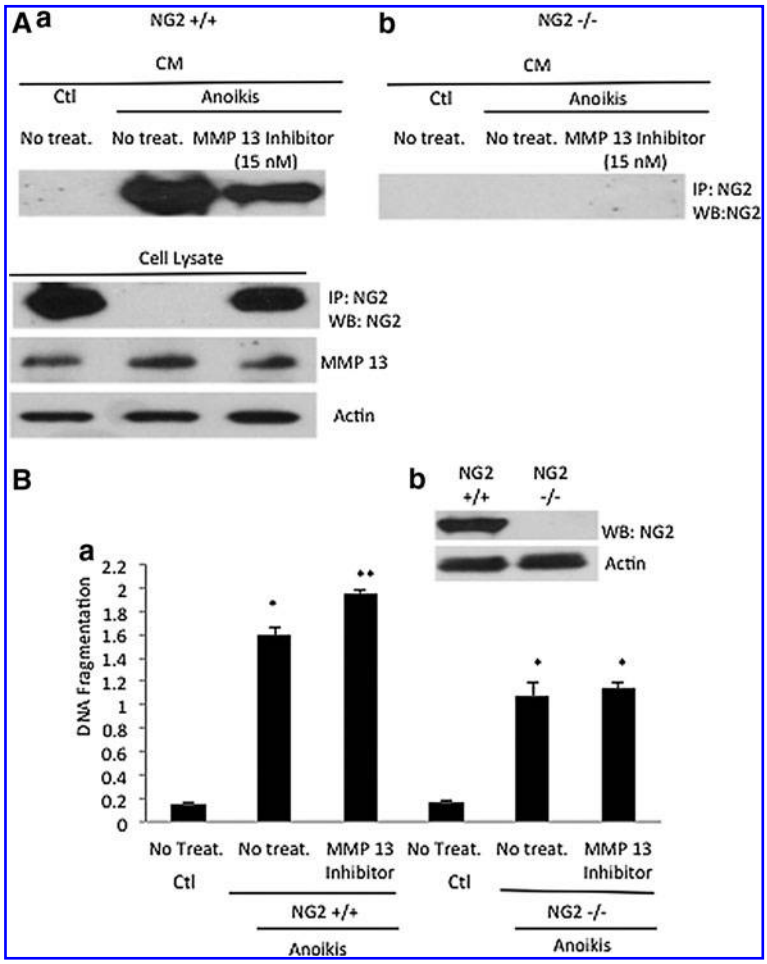

FIG. 4. MMP-13 inhibition did not increase anoikis in NG2-null cells. **(A)** Imunoprecipitation and western blotting of NG2 in **(a)** NG2-positive ($NG2^{+/+}$) and **(b)** NG2-null ($NG2^{-/-}$) aortic smooth muscle cell CM and cell lysates. Cells were pretreated with MMP-13 chemical inhibitor (15 nM) or not treated with inhibitor (no treat.) and then treated under anoikis (fibronectin protein V+H−; 40 μg/mL) or control (Ctl; fibronectin V+H+; 40 μg/mL) conditions for 18 h. **(B)** Cell death ELISA **(a)** assessed under the same condition as in **(A)**. Western blotting **(b)** for NG2 in NG2-positive (NG2 +/+) and NG2-null (NG2 −/−) aortic smooth muscle cells. Values are mean ± SEM. $*p < 0.05$ versus control. $**p < 0.05$ versus anoikis control.

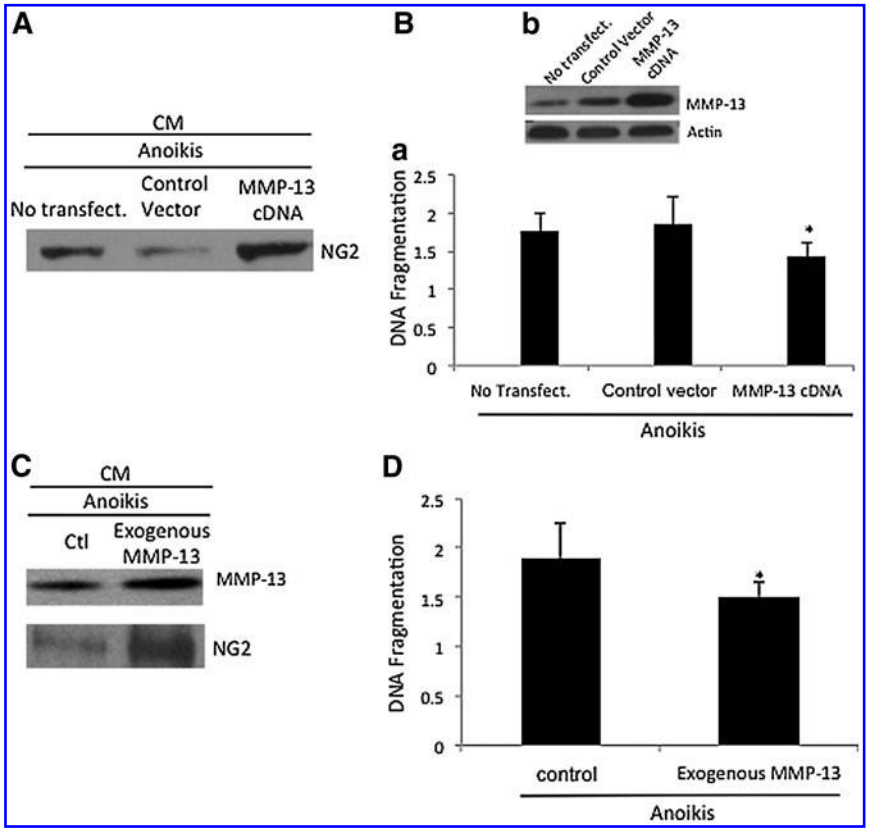

FIG. 5. Overexpression of *MMP-13* inhibits DNA fragmentation under anoikis conditions. **(A)** Western blotting of NG2 in primary human periodontal ligament fibroblast cells transfected with *MMP-13* cDNA or control vector (1.5 µg) and then treated under anoikis conditions (fibronectin protein V+H−; 40 μg/mL) for 18 h. **(B)** Graph of cell death ELISA **(a)** of primary human periodontal ligament fibroblast cells transfected as in **(A)**. Western blot **(b)** confirms *MMP-13* overexpression with transfection. **(C)** Western blotting of NG2 in primary human periodontal ligament fibroblast cells pretreated with exogenous MMP-13 (150 ng/mL) or without (control; Ctl) and then treated under anoikis conditions (fibronectin protein V+H−; 40 μg/mL). **(D)** Cell death ELISA assay assessed under the same conditions as in **(C)**. Values are mean ± SEM. $*p < 0.05$ versus anoikis control.

fibronectin fragments (Joo *et al.*, 2008). Others have shown that fibronectin fragments stimulate *MMP-13* production and promoter activity, which involves PKC and Rac1 signaling pathways (Loeser *et al.*, 2003; Long *et al.*, 2013). Thus, anoikis conditions promote increased MMP-13 expression, which may be regulated by NG2 or fibronectin fragment intracellular signaling to impact/increase *MMP-13* promoter activity. Subsequent MMP-13 activation may be carried out by extracellular urokinase plasminogen activator activity known to be activated in periodontal ligament cells in the context of fibronectin fragments.

Given that periodontal-disease-associated fibronectin fragments induce anoikis and MMP expression, and suppress osteoblastic differentiation in periodontal ligament fibroblasts, the present findings are relevant to periodontal inflammation and periodontal disease pathogenesis (Kapila *et al.*, 1996; Huynh *et al.*, 2002; Jee *et al.*, 2004; Dai *et al.*, 2005; Ghosh *et al.*, 2008, 2010; Joseph *et al.*, 2010; Miao *et al.*, 2011). In this context, our study demonstrates that inhibition of MMP-13 leads to significant increased NG2-mediated cell death in periodontal ligament fibroblasts. One possible mechanism for this induction of cell death in the context of MMP-13 inhibition could be due to decreased phosphorylation of FAK by retention of NG2 on the cell membrane. As shown in our previous study of these cells, anoikis is mediated by *NG2* overexpression due to decreased FAK signaling (Joo *et al.*, 2008). These findings provide valuable information that may help when considering therapeutic approaches for inflammatory diseases, like periodontal disease.

Subantimicrobial or low-dose doxycline (LDD) is known to inhibit collagenase/MMP activity. Since MMP activity, including MMP-13 activity, increases with severity of periodontal disease (Hernandez *et al.*, 2006, 2007; Silva *et al.*, 2008; Hernández Ríos *et al.*, 2009; Leppilahti *et al.*, 2014), LDD has been used to treat periodontal disease. Two long-term studies on patients with periodontal disease showed that daily use of LDD was effective in treating periodontal disease when used alone or as an adjunctive mode of therapy (Crout *et al.*, 1996; Caton *et al.*, 2000). Since LDD is known to inhibit MMP-13 expression and activity *in vivo* (Maher *et al.*, 2014), LDD treatment could compromise periodontal ligament cell survival by promoting NG2 levels and anoikis propagation in these cells. Thus, therapeutics like LDD that target a broad spectrum of collagenases, including MMP-13, need to be carefully evaluated to ensure that MMP inhibitory effects do not negatively impact resident fibroblasts and induce untoward cell death.

MMPs, which have a wide range of substrates, including various collagens and proteoglycans, are responsible for cleavage of ECM molecules under physiological conditions. MMPs can also cleave growth factor receptors, cytokine receptors, notch receptors, and death receptors/ligands to modulate apoptosis (Strand *et al.*, 2004; Chetty *et al.*, 2010; Garg *et al.*, 2010; Wadsworth *et al.*, 2010; Alsaigh *et al.*, 2011; Park *et al.*, 2011; Kim and Jung, 2012). Although these mechanisms have been primarily studied in cancer cell systems, there is also evidence that MMPs regulate cell surface receptor function via cleavage in nontumorigenic cells (Velasco-Loyden *et al.*, 2004; Serratì *et al.*, 2006; Young *et al.*, 2010; Perng *et al.*, 2011). We show that MMP-13 promotes cleavage of the NG2 proteoglycan receptor, and thereby regulates anoikis in periodontal ligament fibroblasts. Further, although putative substrate cleavage consensus sequences for MMP-13 have been reported to include aggregan, collagen II, and collagen IV, it has not been reported whether NG2 contains a cleavage site for MMP-13 (Deng *et al.*, 2000). Our data are the first to suggest that NG2 is a substrate for MMP-13.

In summary, the current data show that increasing retention of NG2 on the membrane by MMP-13 inhibition propagates anoikis in periodontal ligament fibroblasts. In addition, these data show that MMP-13 secreted by fibroblasts is responsible for the shedding of NG2, which subsequently attenuates anoikis. These mechanisms may be important to diseases where anoikis regulation is central, as in inflammatory diseases, including periodontal disease.

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Disclosure Statement

No competing financial interests exist.

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