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Microarray-Based Identification of Aminopeptidase N Target Genes in Keratinocyte Conditioned Medium-Stimulated Dermal Fibroblasts

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ABSTRACT

Of the many processes that affect the outcome of wound repair, epidermal–dermal interactions are essential to extracellular matrix (ECM) remodeling and in particular, soluble factors released by keratinocytes are known to have a direct impact on the production of ECM by dermal fibroblasts. Aminopeptidase N (APN) has recently been proposed as a cell-surface receptor for stratifin and is responsible for the stratifin-mediated matrix metalloproteinase-1 (MMP-1) upregulation in fibroblasts. The present study examines whether modulation of APN gene expression has any impact on the fibroblast ECM gene expression profile. The result reveals that in the presence of keratinocyte-derived soluble factors, transient knockdown of APN in dermal fibroblasts affects the expression of key ECM components such as fibronectin, tenascin-C, MMP-1, MMP-3, and MMP-12. The regulatory effects of APN on fibronectin and selective MMPs appear to be associated with receptor-mediated signal transduction independently of its peptidase activity. On the contrary, inhibition of the APN enzymatic activity by bestatin significantly reduces the tenascin-C expression and enhances the contraction of fibroblast-populated collagen gel, suggesting an activity-dependent regulation of fibroblast contractility by APN. The overall effects of APN on the expression of fibronectin, tenascin-C, and MMPs in fibroblasts propose an important role for APN in the regulation of keratinocyte-mediated ECM remodeling and fibroblast contractile activity. J. Cell. Biochem. 113: 1061–1068, 2012.

KEY WORDS: AMINOPEPTIDASE N; EXTRACELLULAR MATRIX; FIBRONECTIN; TENASCIN-C; MATRIX METALLOPROTEINASE; CONTRACTION

The extracellular matrix (ECM) serves as a scaffold for tissue regeneration and repair by providing structural support to cells, regulating intercellular communication, and maintaining a constant flux of growth factors. In the skin, the composition of the matrix is dynamically modulated by dermal fibroblasts, which continuously synthesize and degrade extracellular molecules and their receptors in response to signaling molecules released by keratinocytes and neighboring cells. Apart from the well-characterized signaling molecules at the epidermal–dermal junction, our group has successfully isolated stratifin (also known as 14-3-3 sigma) which functions as a keratinocyte-releasable stimulating factor of matrix metalloproteinases [Ghahary et al., 2004; Ghaffari et al., 2006]. Stratifin belongs to the 14-3-3 family of phospho-serine/threonine-binding proteins which normally function as intracellular chaperones in signal transduction, cell cycle regulation, molecular transport, and apoptosis [Baldin, 2000; Fu et al., 2000; van Hemert et al., 2001]. Upon release by keratinocytes, stratifin stimulates the expression of matrix metalloproteinase (MMP)-1, -3, -8, and -24 in fibroblasts via the p38 MAPK signaling pathway [Ghahary et al., 2004; Lam et al., 2005; Ghaffari et al., 2006]. In vivo stratifin treatment in a fibrotic rabbit ear model showed improvement of hypertrophic scar in reducing scar thickness and cellularity [Rahmani-Neishaboor et al., 2010]. Further studies indicated that the stratifin-mediated MMP-1 modulation depends on the presence of aminopeptidase N (APN) in fibroblasts [Ghaffari et al., 2010].

APN is a type II transmembrane metalloprotease governing a myriad of biological processes including cell adhesion, motility, differentiation, proliferation, chemokine processing, tumor invasion, and angiogenesis (reviewed in [Mina-Osorio, 2008]). Most of these functions depend on the activity of this membrane-bound enzyme to cleave neutral amino acids from the N terminus of peptides [Bhagwat et al., 2001]. Studies have shown that APN modulates certain cellular processes *independently* of its catalytic

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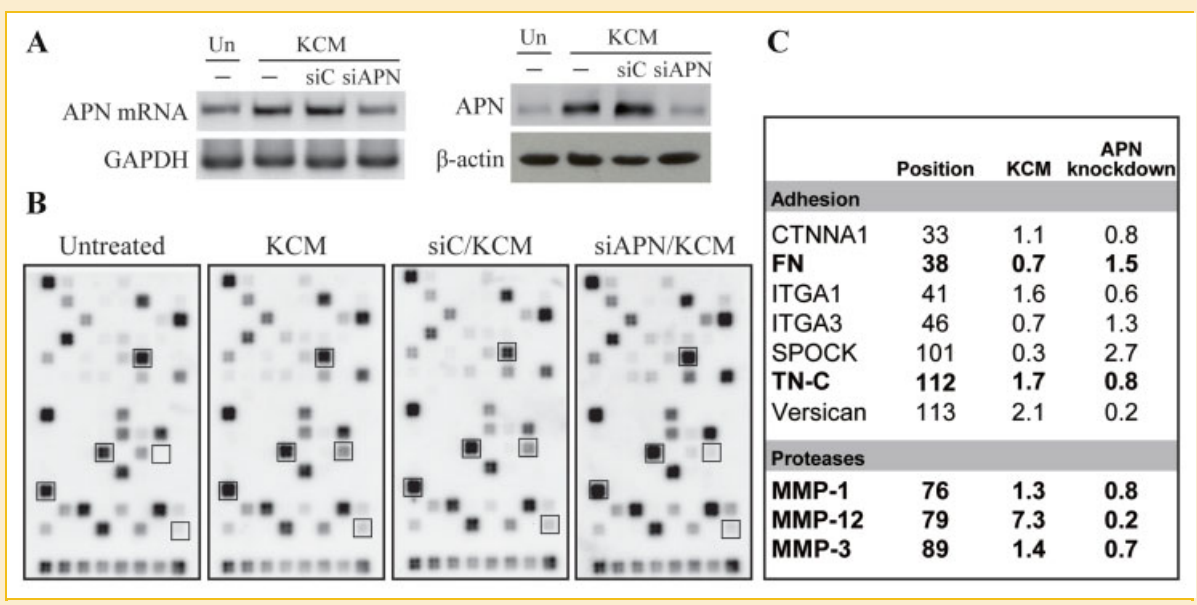
activity and may play a role in signal transduction as a co-regulator of signaling pathways [Santos et al., 2000; Mina-Osorio et al., 2006; Ghaffari et al., 2010]. Thus, while some APN-associated functions can be controlled by modulating its enzymatic activity, others require manipulation at the level of gene expression. An example of such is the paracrine regulation of the cell-surface APN expression on fibroblasts by keratinocyte-derived signals such as stratifin [Lai et al., 2011]. In light of the recent identification of APN as a candidate receptor responsible for stratifin-mediated MMP-1 upregulation [Ghaffari et al., 2010], its induction by stratifin strongly suggests a regulatory role of APN in keratinocyte/fibroblast-mediated ECM remodeling. It was therefore of interest to examine whether APN has any influence on the keratinocyte-mediated regulation of ECM production in fibroblasts. To address this question, we knocked down APN expression by siRNA-mediated gene silencing, and then evaluated by DNA microarray analysis the expression of key ECM components in APN-knocked down fibroblasts upon keratinocyte stimulation. Specifically, this study aimed to identify targets of APN-mediated signal transduction in epidermal–dermal interactions.

RESULTS

ECM GENE EXPRESSION PROFILING IN NORMAL AND APN-KNOCKED DOWN FIBROBLASTS TREATED WITH KERATINOCYTE-CONDITIONED MEDIUM

Epidermal keratinocytes release a myriad of signaling molecules that act as stimuli of cell growth, migration, adhesion, and ECM production for the underlying fibroblasts. To investigate the keratinocyte-mediated changes of ECM gene expression that occur downstream of APN receptor signaling and identify its potential targets in dermal fibroblasts, fibroblasts were transiently transfected with an APN-specific siRNA and exposed to keratinocyte-conditioned medium (KCM) 72 h post-transfection. As shown in Figure 1A, RT-PCR analysis and immunoblot analysis of cells harvested 24 h after KCM addition confirmed that KCM potently stimulated APN expression, and the KCM-induced APN expression was effectively suppressed by an APN-specific siRNA (siAPN) that had been previously shown to reduce its basal expression [Ghaffari et al., 2010]. The introduction of a scramble siRNA (siC) showed no effect on the APN expression.

After confirmation of the siRNA efficiency, a pathway-focused oligonucleotide array was used to monitor the changes of ECM gene expression in fibroblasts. As shown in Figure 1B, arrays were individually incubated with the cDNA of fibroblasts that were untreated, KCM-treated, scramble siRNA-transfected and KCM-treated (siC/KCM), or APN-specific siRNA-transfected and KCM-treated (siAPN/KCM). Of the genes that responded to KCM treatment, those affected by APN knockdown were categorized in Figure 1C according to their main functionality in the skin. These genes are considered candidate targets of APN-mediated signaling in keratinocyte-stimulated fibroblasts because their KCM-induced expression changes were partially or completely offset by APN knockdown. "Position" in panel C refers to positions of the genes in the array layout (Supplementary Material S1). The KCM column shows the fold changes of gene expression as a result of KCM addition to fibroblasts. The APN knockdown column shows the



	Position	KCM	APN knockdown
Adhesion			
CTNNA1	33	1.1	0.8
FN	**38**	**0.7**	**1.5**
ITGA1	41	1.6	0.6
ITGA3	46	0.7	1.3
SPOCK	101	0.3	2.7
TN-C	**112**	**1.7**	**0.8**
Versican	113	2.1	0.2
Proteases			
MMP-1	**76**	**1.3**	**0.8**
MMP-12	**79**	**7.3**	**0.2**
MMP-3	**89**	**1.4**	**0.7**

Fig. 1. Microarray-based ECM gene expression profiling of dermal fibroblasts after APN knockdown and KCM stimulation. Fibroblasts were transfected with a non-silencing siRNA (siC/KCM) or an APN-specific siRNA (siAPN/KCM), and exposed to KCM. Untreated fibroblasts (Un) and KCM-treated fibroblasts (KCM) were used as comparisons. A: APN expression at the mRNA and protein levels. B: Microarray images showing the expression profile of human ECM and adhesion molecules in fibroblasts. C: List of ECM genes whose expression is modulated by both KCM treatment and APN knockdown. The KCM column shows differences in the gene expression between untreated cells and KCM-treated cells; and the APN knockdown column shows differences in gene expression between siC/KCM-treated cells and siAPN/KCM-treated cells. Genes selected for further analysis are shown in bold and their corresponding spots are boxed on the arrays.

levels of gene expression in the siAPN/KCM samples relative to the siC/KCM samples, demonstrating the effect of APN knockdown on the expression of genes regulated by keratinocytes. Genes selected for further analysis are boxed in panel B, and shown in bold in panel C.

Of the genes upregulated in KCM-treated fibroblasts, the induction of MMP-1, MMP-3, MMP-12, versican, tenascin-C (TN-C), integrin alpha 1 (ITGA1), and catenin alpha 1 (CTNNA1) was abrogated by APN knockdown. Of particular interest is the 7.2-fold increase of MMP-12 expression by KCM treatment and the significant reduction that resulted from suppressing the APN expression. Meanwhile, the KCM-mediated inhibition of fibronectin (FN), Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (SPOCK) and integrin alpha 3 (ITGA3) was successfully rescued by APN knockdown.

Previously, we have shown that co-culture of dermal fibroblasts with keratinocytes and addition of stratifin to dermal fibroblasts both led to changes in the expression of MMP-1, MMP-3, and FN [Ghaffari et al., 2006]. In this study, the regulation of these genes as well as of MMP-12 and TN-C by APN was further investigated. Results of the microarray analysis were validated by RT-PCR and shown in Figure 2.

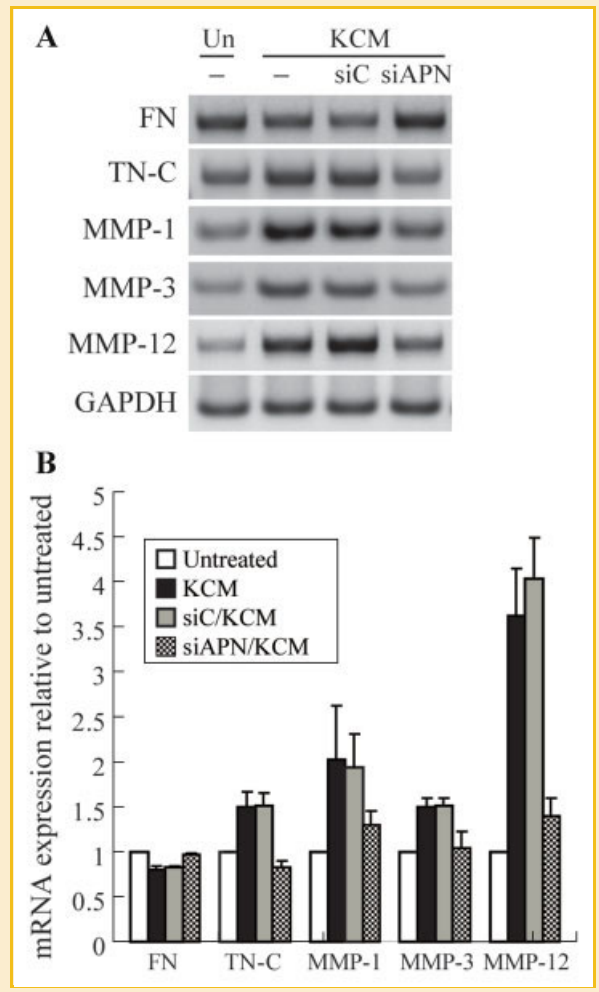


Fig. 2. Validation of the microarray data by RT-PCR analysis. A: Samples of total RNA used in the microarray experiment were reverse transcribed into cDNA and subjected to PCR detection of MMP-1, -3, - 12, FN, and TN-C. B: Their expression levels were quantified by densitometry and normalized by the GAPDH mRNA level. The ratios were calculated, respectively ($n = 3$, $P < 0.03$).

APN MODULATES THE KERATINOCYTE-MEDIATED REGULATION OF MATRIX PROTEASES INDEPENDENTLY OF ITS ENZYMATIC ACTIVITY

To examine whether the changes in gene expression also led to changes in the protein level, cell lysates obtained under the same conditions in the microarray experiment above were subjected to SDS–PAGE and immunoblot analysis (Fig. 3A). The results are presented as relative expression to those in the untreated cells which have been normalized to 1. In correlation with the RT-PCR data and our previous finding, the level of MMP-1 protein was stimulated by KCM treatment. However, the increase of MMP-1 expression was significantly diminished by APN knockdown, leading to an 80% blockage of the keratinocyte-mediated MMP-1 induction. Similarly, it was confirmed that APN suppression abolished the keratinocyte stimulation of MMP-3 at the protein level.

The expression of MMP-12 was similarly stimulated by KCM treatment, although slightly higher in the siC/KCM sample. Despite the slight increase that occurred as a result of the siRNA transfection, suppression of the APN expression significantly blocked the keratinocyte-mediated stimulation of MMP-12. To the authors' knowledge, the expression of MMP-12 (commonly known as macrophage elastase) or its regulation was never studied in detail in dermal fibroblasts. To further confirm this observation, MMP-12 expression was examined in dermal fibroblasts co-cultured with different strains of primary human keratinocytes. As shown in Figure 3B, the presence of keratinocytes significantly increased MMP-12 expression (F/K), validating the influence of epidermal regulation on the protein.

While APN has the structural features of an integral membrane protein, most of the biological functions discovered so far are associated with the catalytic activity of its extracellular domain rather than with its receptor-mediated intracellular signaling. Thus, it is important to determine whether the APN-mediated regulation of MMPs requires its enzymatic activity. Bestatin is a potent inhibitor of aminopeptidase activity and was used to abolish the catalytic activity of APN. Based on results of the enzyme assay performed previously in dermal fibroblasts [Ghaffari et al., 2010], an optimum concentration of 50 μM bestatin was used to effectively inhibit APN enzymatic activity in the current study. As shown in Figure 3D, bestatin treatment alone did not affect the expression of any of MMP-1, -3, and -12, and nor was it able to block the KCM-mediated induction of the MMPs.

APN-MEDIATED DOWNREGULATION OF FN PRODUCTION

Of the adhesion-associated ECM genes affected by APN modulation, FN plays a crucial role in wound healing, and altered FN production has been associated with fibrosis [Craig, 1975; Kischer et al., 1989; Trojanowska et al., 1998]. To validate the microarray data, cells were treated as described above, and their lysates were collected and immunoblotted with an anti-FN antibody (Fig. 4A). Densitometric analysis of the signals showed that the protein expression of FN was reduced by 27% following KCM treatment, and APN knockdown

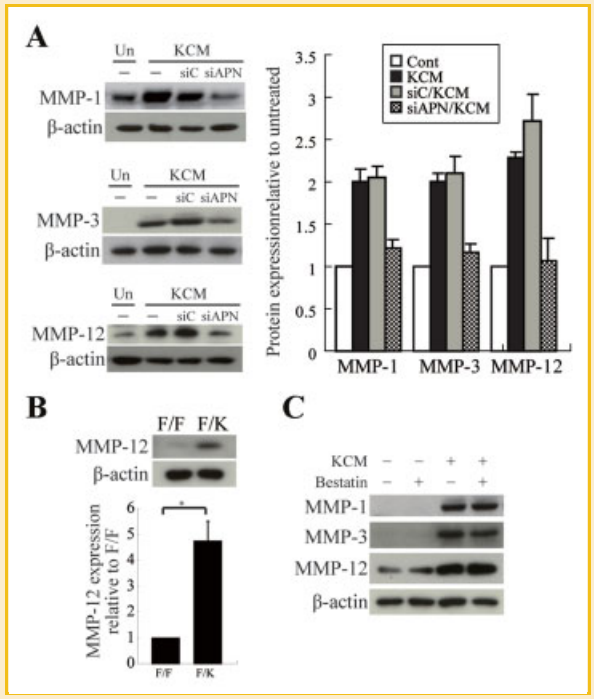


Fig. 3. APN-mediated regulation of matrix proteases. A: Cell lysates of untreated, KCM-, siC/KCM-, siAPN/KCM-treated fibroblasts were collected, and the protein expression of MMP-1, -3, and -12 was examined by immunoblot analysis. Signals from the immunoblots were quantified by densitometry, and normalized by the β-actin level ($n = 3$, $P < 0.02$). B: MMP-12 protein expression in fibroblasts co-cultured with fibroblasts (F/F) or keratinocytes (F/K). The levels of MMP-12 and β-actin were determined by densitometric quantification, and their ratios were calculated ($n = 3$, $P < 0.02$). C: Effect of bestatin on the expression of MMP-1, -3, and -12 in KCM-treated fibroblasts. The cells were treated with 50 μM bestatin for 1 h prior to KCM addition and harvested 24 h later for protein expression analysis.

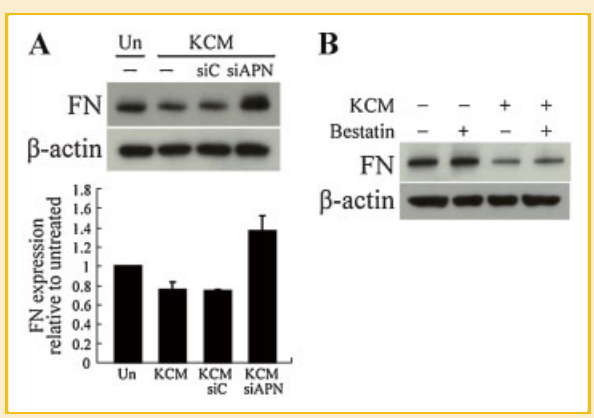


Fig. 4. APN-mediated downregulation of FN production. A: Immunoblot analysis and desitometric quantification of FN protein expression in untreated, KCM-, siC/KCM-, siAPN/KCM-treated fibroblasts. The FN/β-actin ratios were calculated ($n = 3$, $P < 0.03$). B: Effect of bestatin (50 μM) on FN expression in KCM-treated fibroblasts.

completely reversed the inhibition of KCM on FN. In fact, it increased the level of FN expression by 40% when compared to the untreated sample. To examine whether the APN-mediated suppression of FN depends on its enzymatic activity, fibroblasts were treated with KCM in the presence of bestatin. As shown in Figure 4B, inhibition of APN activity did not interfere with the epidermal regulation of FN expression.

APN MODULATES TN-C EXPRESSION IN AN ACTIVITY-DEPENDENT MANNER

Another important adhesion molecule influenced by keratinocyte stimulation is TN-C because of its regulatory role in cell migration and matrix contraction [Midwood and Schwarzbauer, 2002; Midwood et al., 2004]. Microarray analysis showed KCM stimulation of TN-C expression and its reversal by APN knockdown. This was validated by immunoblot analysis using a TN-C-specific antibody (Fig. 5A). Consistent with the microarray data, KCM-treated fibroblasts showed an increase in TN-C expression when compared

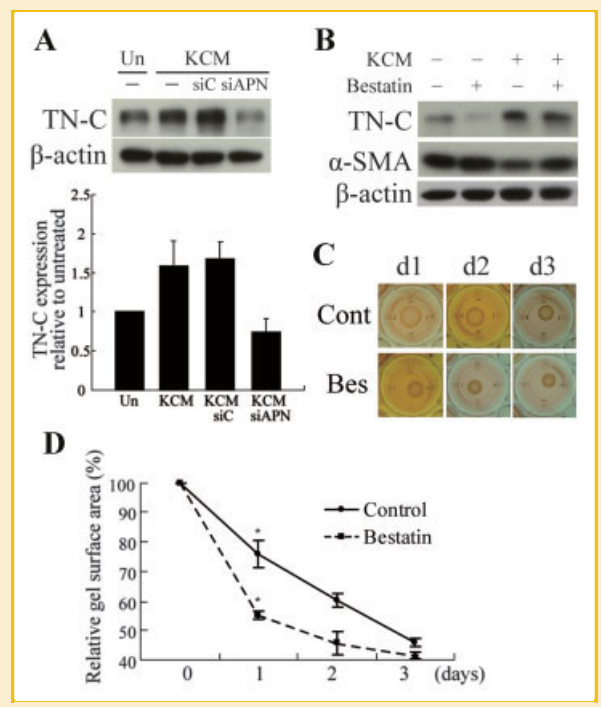


Fig. 5. APN-mediated regulation of TN-C expression and bestatin-induced collagen gel contraction. A: The levels of TN-C and β-actin in untreated, KCM-, siC/KCM-, and siAPN/KCM-treated fibroblasts were determined by densitometric quantification, and their ratios were calculated ($n = 3$, $P < 0.01$). B: Immunoblot analysis of the protein expression of TN-C and α-SMA in dermal fibroblasts after bestatin (50 μM) treatment and 24h KCM incubation. C: Evaluation of the effect of bestatin on fibroblast contractility by collagen gel contraction assay. The surface area of fibroblast-populated collagen gels was measured at days 0, 1, 2, and 3 after gel release in control and bestatin-treated cells. The relative surface area was determined by calculating the surface area of each day's measurement against the surface area of the original gel (panel D, $^{*}P < 0.03$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

with untreated cells, and APN knockdown completely blocked the KCM stimulatory effect, reducing the protein expression of TN-C to the level of the untreated sample.

Based on the role of TN-C in matrix contraction [Midwood and Schwarzbauer, 2002], and a strong body of evidence that shows direct correlation of alpha smooth muscle actin (α-SMA) expression with contraction [Zhang et al., 1996; Hinz et al., 2001], we evaluated α-SMA expression in relation to TN-C expression. KCM treatment in the presence of bestatin showed that bestatin suppressed the basal TN-C expression in fibroblasts but failed to completely abolish the KCM-induced expression of TN-C (Fig. 5B, TN-C). The TN-C expression can be suppressed by inhibiting the catalytic activity of APN; however, the inhibition was not sufficient to circumvent the effect of KCM stimulation. Inhibition of the APN activity also has a direct impact on the α-SMA expression, which showed an inverse pattern to TN-C expression and was increased as a result of bestatin treatment (Fig. 5B, α-SMA).

High level of α-SMA expression is generally associated with enhanced contractile activity [Zhang et al., 1996; Hinz et al., 2001]. Based on our earlier observation that bestatin induces α-SMA expression, we examined the effect of bestatin on the contractile activity of dermal fibroblasts embedded within collagen gels (Fig. 5C). As shown in Figure 5D, the first day measurements demonstrated that the untreated gels (control) shrank 24% while the bestatin-treated gels shrank 45% from their original size. In other words, bestatin has reduced the surface area of fibroblast-populated collagen gel by 21% (relative to the control) during the first 24 h of treatment, and the inhibitory effect gradually wore off as the incubation period extended to 48 and 72 h.

DISCUSSION

Stratifin is an ECM-modulating factor released by keratinocytes, and the recent identification of APN as a fibroblast surface receptor for stratifin has raised attention to its potential role in regulation of cell behavior and matrix remodeling in the dermis. Although some physiological substrates are known, very few downstream targets have been identified for APN because of its short cytoplasmic domain and unknown binding partners. This is the first time that APN has been shown to regulate ECM proteins. Of the genes that responded to APN modulation in KCM-stimulated fibroblasts, the adhesion-associated gene (fibronectin), matrix-degrading proteases (MMPs), and contraction-associated gene (tenascin-C) are of special interest due to their implication in tissue remodeling during wound healing. More importantly, these candidate targets are involved in biological processes that are consistent with APN functions established from studies using cross-linking antibodies and activity inhibitors, such as tumor metastasis and ECM degradation [Saiki et al., 1993; Fujii et al., 1996; Wulfaenger et al., 2008].

The present study discovered that MMP-12 is present in dermal fibroblasts and its expression is under the influence of epidermal keratinocytes. Although commonly known as macrophage elastase, MMP-12 is also expressed in non-immune cells such as vascular fibroblasts, smooth muscle cells, and corneal fibroblasts [Mahajan et al., 2002; Woodside et al., 2003]. MMP-12 is the most potent MMP against elastin [Shapiro, 1998], and can degrade many other ECM components because of its ability to initiate a cascade of proteolytic events by activating pro-MMP-2 and pro-MMP-3 [Matsumoto et al., 1998]. While the current study addressed APN-mediated regulation of MMP-12 only in dermal fibroblasts, it is likely to also occur in immune cells as suggested by the prominent role of APN in T-cell response and cytokine production [Kanayama et al., 1995; Shimizu et al., 2002; Reinhold et al., 2006; Proost et al., 2007a].

TN-C shows a temporo-spatial distribution in human adult skin and is specifically expressed near the wound edge within 24 h of injury [Mackie et al., 1988; Betz et al., 1993]. Given the dynamic and transient nature of TN-C expression, tremendous efforts have been made to study the mode of TN-C regulation, in particular, to identify molecules and regulatory pathways that govern the local expression of TN-C. Here, we showed that TN-C expression is stimulated by KCM which contains a plethora of keratinocyte-released signaling molecules, and that the keratinocyte-mediated TN-C stimulation is abolished in fibroblasts with suppressed APN expression. The fact that the fibroblast expression of TN-C can be modulated by epidermal keratinocytes may explain the reported increase of TN-C expression in wound-edge fibroblasts [Mackie et al., 1988; Betz et al., 1993]. In an open wound, as epithelial cells migrate towards the site of injury, fibroblasts at the wound edge become exposed to epithelial cells, and increase their TN-C expression in receipt of signals released by these cells.

In addition to its anti-adhesive and pro-migratory role, TN-C inhibits matrix contraction through downregulation of focal adhesion kinase (FAK) phosphorylation [Midwood and Schwarzbauer, 2002; Midwood et al., 2004]. Induction of TN-C at the early stage of wound healing represses premature contraction, while persistent TN-C expression after granulation tissue formation in embryonic wound or oral mucosal wound is thought to prevent excessive contraction and thus scar formation [Mackie et al., 1988; Latijnhouwers et al., 1996; Wong et al., 2009]. Inhibition of the APN enzymatic activity by bestatin was not sufficient to abrogate the effect of KCM stimulation on TN-C; however when used alone, bestatin clearly suppressed the basal expression of TN-C in fibroblasts, suggesting that APN may be involved in modulating fibroblast contractile activity. This was confirmed by the elevated α-SMA expression in bestatin-treated fibroblasts. As a transmembrane protease, APN is known to cleave various cytokines and growth factors, and in most situations the cleavage causes inactivation of bioactive molecules [Hoffmann et al., 1993; Xu et al., 1995a; Fortin et al., 2005; Proost et al., 2007b; Danziger, 2008]. It is possible that bestatin suppresses the enzymatic activity of cell-surface APN to cleave and inactivate an unknown stimulating factor in the extracellular environment, thereby causing a reduction of TN-C production in fibroblasts. The importance of APN in regulation of fibroblast contractility was further evaluated using a fibroblast-populated collagen gel contraction assay which demonstrated that bestatin promotes contraction. The same phenomenon is observed in the gastric system, where aminopeptidase inhibitors have been proven to potentiate the enkephalin-stimulated contraction of gastric smooth muscle cells [Menozzi et al., 1991a]. Under physiological conditions, APN degrades Leu- and Met-enkephalins, thereby limiting the number of available peptides to activate

receptors [Matsas et al., 1985; Giros et al., 1986; Xu et al., 1995b]. Thus, inhibition of APN activity significantly increased the potency of Met-enkephalin to induce contraction [Menozzi et al., 1991b].

In contrast to TN-C modulation, activity inhibition had no effect on the expression of FN or MMPs, suggesting that the APN-mediated regulation of these genes is likely to involve intracellular signaling events associated with its receptor function. This hypothesis is further supported by our previous finding on stratifin-mediated ECM modulation, which showed that stratifin stimulates MMP-1 expression via the p38/MAPK pathway in an APN-dependent manner and that the stimulatory effect is unaffected by suppression of the APN catalytic activity [Lam et al., 2005; Ghaffari et al., 2010].

In summary, the present study showed that in the presence of a virtually complete repertoire of keratinocyte-derived growth factors and cytokines, the fibroblast production of certain ECM components relies on the availability of APN. The additional level of regulation conferred by the cell-surface APN receptor on fibroblasts is critical for the transmembrane signaling of certain keratinocyte-derived stimuli, including those that influence the expression of FN, MMPs, and TN-C. Accordingly, dysregulation of APN may result in a fibrotic phenotype due to over-accumulation of ECM and reduced matrix-degrading activity. In line with the proposed effect of its ligand stratifin in ameliorating hypertrophic scarring [Rahmani-Neishaboor et al., 2010], the direct impact of APN on ECM gene expression makes it an ideal therapeutic target in wound healing.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Skin punch biopsies were obtained with informed consents from patients undergoing elective circumcision. The study was approved by the University of British Columbia Hospital Human Ethics Committee and conducted according to the Declaration of Helsinki Principles. The detailed protocol of harvesting fibroblasts and keratinocytes from skin has been described previously [Ghahary et al., 2005]. Fibroblasts were grown in DMEM with 10% FBS and keratinocytes in KSFM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with bovine pituitary extract (50 μg/ml) and EGF (0.2 μg/ml). Fibroblasts of passages 3–6 and keratinocytes of passages 3–5 were used in this study. KCM was collected from keratinocytes cultured in KSFM without supplements. Bestatin hydrochloride (Sigma Chemicals, Oakville, ON, Canada) was used as a competitive inhibitor of APN enzymatic activity.

siRNA KNOCKDOWN OF APN

For APN knockdown, an siRNA oligonucleotide (Hs_ANPEP_5 FlexiTube siRNA; SI02780211) purchased from Qiagen (Valencia, CA) was used. The APN-specific siRNA oligonucleotide targets the sequence of CCGAAATGCCACACTGGTCAA at positions 2740–2760 of the human ANPEP sequence (NM_001150). A non-silencing siRNA with the same GC content as the APN siRNA was used as a negative control. HiPerfect transfection reagent was used according to the manufacturer's recommendations (Qiagen). Fibroblasts were seeded at 1×10^5 cells/well and transfected with 25 nM of the siRNA oligonucleotide. The medium was replaced 24 h later and the cells were treated with KCM at 72 h post-transfection.

GENE EXPRESSION ANALYSIS BY ECM-SPECIFIC MICROARRAY

To examine the impact of APN modulation on keratinocyte-regulated ECM gene expression in dermal fibroblasts, Oligo GEArray® pathway-specific gene expression arrays were purchased from SuperArray Bioscience Corporation (Fredrick, MD). Each gene array consists of 114 genes of human ECM and adhesion molecules involved in cell adhesion, ECM deposition, and degradation, as well as sequences for loading control such as β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The arrays were used according to the manufacturer's instructions. In brief, total RNA was isolated from untreated cells (Un) as well as cells subjected to KCM treatment (KCM), scramble siRNA transfection and KCM (siC/KCM), and APN-specific siRNA transfection and KCM (siAPN/KCM) using the RNeasy Mini Kit (Qiagen). cDNA was then prepared from the total RNA using MMLV reverse transcriptase, biotinylated with Biotin-16-dUTP (Roche, Indianapolis, IN), and hybridized to a positively charged nylon membrane containing the arrayed DNA. The arrays were visualized using the chemiluminescent detection system provided (SuperArray Bioscience Corporation). Loading was adjusted based on the intensity of hybridization signals to the housekeeping gene, GAPDH, and then gene expression was quantified by densitometric analysis using the ImageJ software available from NIH.

REVERSED TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

To validate the changes of gene expression observed in the microarray analysis, RT-PCR was conducted using total RNA isolated from cells treated under the same conditions utilized in the microarray experiment. Total RNA was reverse transcribed into cDNA using SuperScript First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA). PCR analysis of the samples was carried out using the prepared cDNA as template, and the primers listed below.

FN:	CAGACCTATCCAAGCTCAAGT
	GCCTTTGATGGTGTAGGAGTT
TN-C:	GGTACAGTGGGACAGCAGGT
	GGGCTGGTTGTATTGATGCT
MMP-1:	GATGTGGAGTGCCTGATGTG
	TGCTTGACCCTCAGAGACCT
MMP-3:	CCTCAGGAAGCTTGAACCTG
	GGGAAACCTAGGGTGTGGAT
MMP-12:	ACACATTTCGCCTCTCTGCT
	CCAGGGTCCATCATCTGTCT
GAPDH:	GAAGGTGAAGGTCGGAGTC
	GAAGATGGTGATGGGATTTC

The PCR amplification products were subjected to agarose gel electrophoresis and visualized under UV light. The levels of intensity were quantified using ImageJ and normalized to the GAPDH mRNA level.

COLLAGEN GEL CONTRACTION ASSAY

Analysis of fibroblast-populated collagen gel contraction was performed as described [Lenga et al., 2008] in collagen gels containing 200,000 cells per gel treated with bestatin. Changes in surface area were measured every 24 h for 3 days. Ultrapure bovine collagen solution (3 mg/ml) was used (Sigma Chemicals, Oakville, ON, Canada).

IMMUNOBLOT ANALYSIS

Lysates of dermal fibroblasts subjected to siRNA transfection followed by KCM treatment were collected. Total protein concentration was determined using Bradford protein assay. Fixed amounts of proteins of each sample were subjected to SDS–PAGE and immunoblot analysis as previously described [Lai et al., 2010]. The following antibodies were used: mouse monoclonal anti-APN/clone 3D8 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal anti-FN/clone H300 (Santa Cruz Biotechnology Inc.), mouse monoclonal anti-procollagen type I/clone SP1.D8 (Developmental Studies Hybridoma Bank, maintained by the University of Iowa, Department of Biological Science, Iowa City, IA), rabbit monoclonal anti-MMP-1 (Epitomics, Burlingame, CA), rabbit monoclonal anti-MMP-3 (Epitomics, Burlingame, CA), rabbit polyclonal anti-MMP-12 (Millipore, Billerica, MA), rabbit polyclonal anti-TN-C (Santa Cruz Biotechnology Inc.), rabbit monoclonal anti-α-SMA (Epitomics), or mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology Inc.).

STATISTICAL ANALYSIS

Data were expressed as mean ± SD. The ANOVA's test was used to compare the mean values between different treatments in the siRNA experiment. Student's *t*-test was used for analysis of the co-culture MMP-12 expression and collagen gel contraction. *P*-values of < 0.05 were considered statistically significant in this study.

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