Published in final edited form as:

J Mol Cell Cardiol. 2010 October ; 49(4): 655–663. doi:10.1016/j.yjmcc.2010.05.007.

EMMPRIN activates multiple transcription factor pathways in cardiomyocytes, and induces interleukin-18 expression via Rac1-dependent PI3K/Akt/IKK/NF- κ B and MKK7/JNK/AP-1 signaling

Balachandar Venkatesan^{a,b}, Anthony J. Valente^c, Sumanth D. Prabhu^d, Prakashsrinivasan Shanmugam^b, Patrice Delafontaine^b, and Bysani Chandrasekar^{a,b,*}

^a Research Service, Southeast Louisiana Veterans Health Care System, New Orleans, LA 70161

^b Heart and Vascular Institute, Tulane University School of Medicine, New Orleans, LA 70112

^c Department of Medicine, University of Texas Health Science Center, San Antonio, TX 78229

^d Institute of Molecular Cardiology, Department of Medicine, University of Louisville, Louisville, KY 40292, and the Medical Service, Louisville Veterans Affairs Medical Center, Louisville, KY 40206

Abstract

The transmembrane glycoprotein extracellular matrix metalloproteinase inducer (EMMPRIN), and the pleiotropic proinflammatory cytokine interleukin (IL)-18, play critical roles in myocardial remodeling, by inducing matrix degrading metalloproteinases (MMPs). Previously we showed that IL-18 induces EMMPRIN expression in cardiomyocytes via MyD88/IRAK4/TRAF6/JNK-dependent Sp1 activation. Here in reciprocal studies we demonstrate that EMMPRIN is a potent inducer of IL-18 transcription, protein expression and protein secretion in primary mouse cardiomyocytes. We show for the first time that EMMPRIN stimulates the activation of NF- κ B, AP-1, CREB, and ATF-2 in cardiomyocytes, and induces IL-18 expression via Rac1-dependent PI3K/Akt/IKK/NF- κ B and MKK7/JNK/AP-1 signaling. Moreover, EMMPRIN induces robust time-dependent induction of various MMP mRNA. EMMPRIN also induces the mRNA of TIMPs 1 and 3, but in a delayed fashion. These results suggest that IL-18-induced EMMPRIN expression may favor net MMP expression and ECM destruction, and thus identify both as potential therapeutic targets in countering adverse myocardial remodeling.

Keywords

Proinflammatory cytokines; Matrix metalloproteinases; Myocardial remodeling; Cardiac hypertrophy; Myocardial failure

* Address for correspondence: Bysani Chandrasekar, DVM, Ph.D., Heart and Vascular Institute, Tulane University School of Medicine, 1430 Tulane Avenue, SL-48, New Orleans, LA 70112, Tel.: 504-988-3034, Fax: 504-988-4237, bchandra@tulane.edu.

DISCLOSURE: None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

A balance between extracellular matrix (ECM) deposition and matrix destruction is important for maintaining normal myocardial architecture and integrity. This balance is regulated by matrix degrading metalloproteinases (MMPs) and their inhibitors the tissue inhibitors of matrix metalloproteinases (TIMPs). Under pathological conditions, increased expression of MMPs can induce excess ECM breakdown, and lead to adverse myocardial remodeling. In addition to various proinflammatory cytokines expressed during myocardial injury and inflammation, EMMPRIN (extracellular matrix metalloproteinase inducer) has also been shown to induce the expression of diverse MMPs in cell type-specific manner [1,2]. EMMPRIN was first identified in tumor cells, where its high level of expression is thought to induce MMPs in peritumoral or stromal cells, thus effecting ECM destruction and tumor metastasis [1,2]. Later, it was discovered that EMMPRIN is actually expressed at low levels in various organs under normal physiological conditions, and its induction in pathological conditions is associated with adverse tissue remodeling [1–3].

EMMPRIN is thought to exert its biological effects in a number of ways. As an integral membrane protein, intercellular homophilic EMMPRIN-EMMPRIN interaction has been shown to induce MMPs expression in adjacent cells [4]. Further, the presence of leucine zipper-like sequences, and a positively charged glutamic acid in its hydrophobic transmembranous region implies that EMMPRIN may also interact with non-EMMPRIN transmembrane proteins and activate diverse cellular signaling pathways [1,3]. Following its cleavage from cell surface by MMPs, soluble EMMPRIN may act distally in a paracrine manner [5]. Its critical role in numerous physiological functions is further emphasized in EMMPRIN-null mice, which show various developmental defects, including poor implantation of the embryos, sterility, blindness, and memory and sensory defects [6,7].

Recently, it was demonstrated that EMMPRIN levels are elevated in monocytes in patients with acute myocardial infarction [8]. Further, treatment of monocytes isolated from normal subjects with soluble EMMPRIN activated NF- κ B and induced κ B-responsive cytokine gene expression [9], implying that monocyte-derived EMMPRIN could be involved in adverse remodeling via induction of MMPs and cytokines in myocardial constituent cells. In fact, cardiomyocytes and smooth muscle cells also express EMMPRIN [10,11]. Of note, EMMPRIN expression levels are increased in hypertrophied left ventricular tissue in patients undergoing aortic valve replacement [12], and in patients with end-stage cardiomyopathy [1,13]. Importantly, transgenic overexpression of EMMPRIN in a cardiac-specific manner results in adverse myocardial remodeling and failure [14]. These transgenic mice express increased levels of myocardial MMP-2 and membrane type-1 MMP (MT1-MMP or MMP14), and show fibrosis and adverse LV remodeling [14]. EMMPRIN has also been shown to induce the expression of MMPs 1 and 9 in fibroblasts [14], and all of these MMPs play a role in adverse remodeling. Interestingly, it is reported that EMMPRIN fails to modulate tissue inhibitors of MMPs (TIMPs; [2]), thus its upregulation would favor unopposed MMP expression, activation, and ECM destruction.

The proinflammatory cytokine IL-18, that belongs to the IL-1 family of pleiotropic cytokines, is synthesized as an inactive, non-glycosylated precursor. Activated proteolytically by caspase-1, IL-18 binds to cell surface receptors comprised of a and β transmembrane protein subunits, which heterodimerize upon IL-18 binding, and activate an intricate network of intracellular signaling pathways [15]. Encoded by a separate gene, the IL-18 binding protein (IL-18BP) lacks the extracellular ligand-binding domain of the IL-18 receptor, but binds IL-18 with high specificity and affinity, and neutralizes its biological activity [15]. All four major myocardial constituent cells express IL-18, but respond to IL-18 in a cell type-specific manner. In endothelial cells, it induces the expression of cytokines,

and stimulates apoptosis via NF- κ B-dependent PTEN activation [16]. It stimulates SMC migration in part via MMP9 [17], and its administration induces fibrotic changes in fibroblasts [18]. It exerts pro-growth effects in cardiomyocytes [19]. Together, these *in vitro* results suggest that IL-18 is a pleiotropic cytokine with pro-apoptotic, pro-mitogenic, pro-hypertrophic, and proinflammatory effects. In *vivo*, it is upregulated in various models of myocardial inflammation and injury. We have previously demonstrated that neutralization of IL-18 attenuates experimental myocardial ischemia/reperfusion injury [20], and lack of IL-18 expression in IL-18 null mice inhibits pressure overload-induced myocardial hypertrophy [21]. More recently, administration of mesenchymal stem cells expressing IL-18 binding protein, the natural inhibitor of IL-18, has been shown to improve myocardial protection following ischemia and infarction [22]. More critically, its expression is increased in patients with myocardial infarction, hypertrophy and failure [23]. These studies imply that IL-18 plays a causal role in various models of cardiac injury and inflammation. Since both EMMPRIN and IL-18 expression is elevated in myocardial injury, hypertrophy, and failure, and may induce cytokine and MMPs expression, and mediate adverse remodeling, we investigated whether EMMPRIN induces IL-18, as well as the expression of various MMPs and TIMPs in cardiomyocytes and determined the underlying molecular mechanisms.

2. Materials and methods

2.1. Adult and Neonatal Mouse Cardiomyocytes

Calcium-tolerant adult mouse cardiomyocytes (ACM) were isolated from 3 month-old male C57Bl/6 mice by a modified Langendorff perfusion and collagenase digestion technique, and has been previously described [24]. Since ACM are extremely difficult to transfect using standard transfection protocols, we used neonatal mouse cardiomyocytes (NMCM), which show relatively higher transfection efficiency (~33% with only 9% cell death as determined using the pEGFP-N1 vector; Clontech, Mountain View, CA) to investigate the regulation of the *IL18* 5' *cis* regulatory region. NMCM were isolated from 1-to 3-day-old neonatal mice (C57Bl/6 background) as previously described [20].

2.2. Adeno and lentiviral transduction

Cardiomyocytes were infected at ambient temperature with adenoviruses (Supplementary file) in PBS at the indicated multiplicities of infection (MOI; [10, 20, 24]). After 1 h, the adenovirus was replaced with culture media supplemented with 0.5% BSA. Assays were carried out 24 h later. The transfection efficiency with the adenoviral vectors was near 100%, and infection with the adenoviral vectors at indicated MOI had no significant effect on cardiomyocyte shape, adherence, and viability.

2.3. Cell death detection ELISA

Cardiomyocytes exposed to EMMPRIN for 24 h were harvested and analyzed for mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by ELISA (Cell Death Detection ELISA^{PLUS} kit, Roche Applied Science). Doxorubicin, a potent anti-neoplastic drug that induces cardiomyocyte death, served as a positive control (1 μ M for 24 h; [24]).

2.4. Transcription factor activation

Nuclear extracts—Nuclear extracts were prepared using the Panomics Nuclear Extraction Kit according to the manufacturer's instructions (#AY2002; Panomics/Affymetrix, Freemont, CA; [20]).

Protein-DNA interaction array—Protein-DNA interactions were analyzed using the TranSignal Protein/DNA Array 1 (PD array 1, Panomics/Affymetrix) essentially as

described by Imam, et al ([25]; A detailed description of the methodology and the TFs assayed are provided in the Supplementary file and Fig. S2).

Regulation of EMMPRIN-mediated transcription factor activation was confirmed by a highly-sensitive signaling profiling ELISA (TransFactor kits (BD Mercury TransFactor Profiling Assay). This assay profiles NF- κ B-p65, p50, c-Rel, c-Fos, FosB, cJun, JunD, CREB, ATF2, Sp-1, and STAT1, and has a 10-fold higher sensitivity than traditional electrophoretic mobility shift assays with fewer false negative results (sensitivity for NF- κ B >0.3 nM; [26]). Cardiomyocytes were treated with EMMPRIN for 2 h, and 25 μ g of nuclear extracts were used. After the addition of chromogen, absorbance at 650 nm was measured in a SPECTRAMax Plus spectrophotometer.

The formation of NF- κ B protein-DNA complexes and the subunit composition were also analyzed by an ELISA (TransAMTM TF ELISA kits; #43296; Active Motif, Carlsbad, CA; [10,20,24]). Activation of NF- κ B was also confirmed by a reporter assay using adenoviral transduction of an NF- κ B reporter vector (Ad. NF- κ B-Luc, MOI 50) as described previously [20]. Ad. AP-1-Luc was also previously described. Ad. MCS-Luc (MOI 50) served as negative control, and Ad. β -gal (MOI 50) served as an internal control. β -Galactosidase activity in cell extracts was determined using a luminescent β -galactosidase detection kit II (BD Biosciences), and the results are expressed as the ratio of firefly luciferase to β -galactosidase activity measured in relative light units. Activation of NF- κ B was further confirmed by immunoblotting using anti-p65 antibodies. The binding of AP-1 and NF- κ B to the *IL18* 5' *cis* regulatory region *in vivo* was investigated by ChIP assays [20].

2.5. Promoter reporter activity

IL18 promoter analysis. The murine *IL18* gene contains two TATA-less functional regions upstream of exon 1 (inducible; -2505 to +61 nt) and exon 2 (basal; -540 to +61 nt), and both regions contain NF- κ B and AP-1 response elements [27,28]. We performed transient transfection assays using the inducible promoter-reporter construct (-2505 to +61 nt; pIL18-Luc) and analyzed its inducibility by EMMPRIN. NMCM were transfected with 3 μ g of pIL18-Luc or pGL3-Basic (vector control) together with *Renilla* luciferase vector (100 ng, pRL-TK, Promega Corporation, Madison, WI) to control for transfection efficiency. Twenty-four hours later, cells were treated with EMMPRIN. Firefly and *Renilla* luciferase activities were analyzed at 12 h post-treatment.

2.6. mRNA expression

IL-18 mRNA expression was analyzed by reverse transcription (RT) followed by real time quantitative PCR (qPCR) (see Supplementary file for a detailed protocol). The expression of IL-18 mRNA was confirmed by Northern blotting. 28S rRNA served as a loading control. Expression of MMPs and TIMPs at 1 and 24 h following EMMPRIN treatment was analyzed using a qPCR-based array (PAMM-013; SABiosciences, Frederick, MD 21703, USA) that detects 13 MMPs (MMP 1, 2, 3, 7, 8, 9, 10, 11, 12, 13, 14 [membrane type 1-MMP or MT1-MMP], 15 [MT2-MMP], 16 [MT3-MMP]) and 3 TIMPs (TIMP 1, 2, 3). Expression of MMP2 and 9 was also confirmed by RT-qPCR using TaqMan[®] gene expression assay, with Actin serving as an invariant control.

2.7. Analysis of protein expression

Preparation of protein homogenates, immunoblotting, detection of the immunoreactive bands by enhanced chemiluminescence (ECL Plus; GE Healthcare), and their quantification by densitometry were described previously [17,19,27]. IL-18 levels in culture supernatants were quantified by an ELISA (Mouse IL-18 ELISA kit, code no. 7625; R & D Systems). The sensitivity of the assay is 25 pg/ml.

2.8. Rac1, MKK7, PI3K, and IKK activation assays

Rac1 activity was assayed according to the manufacturer's protocol using an EZ-detect Rac1 activation kit (Pierce, Rockford, Ill), and has been previously described [29]. Mitogen-activated protein kinase kinase 7 (MKK7) activity was analyzed by immune-complex kinase assays using GST-cJun as the substrate. PI3K and IKK β activities were quantified as described previously [10,20,24].

2.9. Statistical Analysis

Comparisons between controls and various treatments were performed by analysis of variance with post hoc Dunnett's *t* tests. All assays were performed at least three times, and the error bars in the figures indicate the S.E. Though a representative immunoblot is shown in the main figures, changes in protein/phosphorylation levels from three independent experiments were semi-quantified densitometrically, and are included in the corresponding Supplementary figures along with statistical analyses.

3. Results

3.1. EMMPRIN is a potent specific inducer of IL-18 expression in cardiomyocytes

Adult mouse cardiomyocytes (ACM) were incubated with 0 – 10 ng/ml recombinant EMMPRIN, and IL-18 mRNA expression determined by RT-qPCR. EMMPRIN induced IL-18 expression in a dose-dependent manner, with peak levels (~4-fold increase) detected at 1 ng/ml (Fig. 1A). Adenoviral transduction of mutant EMMPRIN or incubation with EMMPRIN neutralizing antibodies prior to EMMPRIN addition significantly reduced the response (Fig. 1B), whereas their respective controls Ad. GFP and normal IgG, did not (Fig. 1B), demonstrating the specificity for EMMPRIN. These results were supported by Northern blot analysis, which also demonstrated the time-dependent induction of IL-18 by EMMPRIN (Fig. 1C). EMMPRIN induced IL-18 protein expression in a time-dependent manner (Fig. 1D), and stimulated its secretion (Fig. 1E). Since the recombinant EMMPRIN contains low levels of endotoxin (<1.0 EU per microgram of the protein as determined by the LAL method; manufacturer's data sheet), and LPS is known to induce EMMPRIN, we investigated whether this low level of contaminating endotoxin may have contributed to the induction of IL-18 expression. ACM were incubated with EMMPRIN in the presence of 10 μ g/ml polymyxin B, an inhibitor of endotoxin activity. Similar levels of EMMPRIN-induced IL-18 protein were observed in the cells whether polymyxin B was present or not (Fig. 1F), strongly suggesting that the observed response was due to EMMPRIN and not to endotoxin.

3.2. EMMPRIN activates several cis regulatory elements in adult mouse cardiomyocytes (ACM)

EMMPRIN has recently been shown to activate NF- κ B and induce various κ B-responsive genes in monocytes [9]. Since EMMPRIN induced IL-18 expression (Fig. 1), and as the regulatory region of the *IL18* gene contains several potential transcription factor-binding sites, including NF- κ B [28], we investigated the trans-acting factors that can be induced by EMMPRIN in cardiomyocytes. ACM were incubated with EMMPRIN (1 ng/ml) for 2 h, and nuclear extracts analyzed for transcription factor (TF) activation/inactivation using an array that detects 56 transcription factor elements simultaneously. Confirming earlier reports of NF- κ B activation in monocytes [9], treatment with EMMPRIN markedly increased NF- κ B DNA binding in cardiomyocytes (Fig. 2A; compare lanes G7, G8 in EMMPRIN and control arrays). EMMPRIN also activated activator protein-1 (AP-1; compare A1, A2), cyclic AMP-response element binding protein (CREB; compare C1, C2), and AP-2 (compare A3, A4). Purity and equal loading of nuclear extracts was confirmed by immunoblotting in the right hand panel (Fig. 2A). Using a highly sensitive signal profiling ELISA (TransFactor kits (BD

Mercury TransFactor Profiling Assay), we confirmed the activation of NF- κ B (comprised of p65 and p50), AP-1 (cFos, cJun, FosB) and CREB, and also identified the CREB family member activating transcription factor-2 (ATF-2) as an additional transcription factor induced in cardiomyocytes by EMMPRIN (Fig. 2B). In supporting studies, we used reporter assays, immunoblotting, and TransAM TF ELISA to confirm activations of NF- κ B, AP-1, CREB and ATF-2. EMMPRIN induced a significant increase in NF- κ B-dependent reporter gene activation (Fig. 2C) and p65 nuclear translocation (Fig. 2D, immunoblotting) and p65 binding to the *IL18* promoter *in vivo* (Fig. 2E). Similarly, EMMPRIN increased AP-1-dependent reporter gene activation (Fig. 2F) and *in vivo* binding to the *IL18* promoter (Fig. 2G). Further, EMMPRIN induced CREB and ATF-2 activation as evidenced by a time-dependent increase in phospho-CREB (Ser133; Fig. 2H) and phospho-ATF-2 (Thr69/Thr71; Fig. 2I) levels in the nuclear protein extracts.

3.3. EMMPRIN induces IL-18 transcription via NF- κ B and AP-1, but not CREB

We next investigated whether these *cis* regulatory elements mediate EMMPRIN-induced IL-18 expression. Since ACM are extremely difficult to transfect, we used NMCM (transfection efficiency ~33%) in these promoter-reporter studies. EMMPRIN stimulated a ~4-fold increase in the *IL18* promoter reporter gene activity (Fig. 3A), and this was inhibited by forced expression of dnp65, kdIKK β , and dncJun (Fig. 3A). Similarly, EMMPRIN-induced IL-18 mRNA (Fig. 3B) and protein expression (Figs. 3C and 3D) were also inhibited by these dominant negative signaling intermediates. In contrast, forced expression of dominant negative mutant CREB (KCREB), while inhibiting EMMPRIN-induced CREB phosphorylation (Fig. 3E), failed to inhibit EMMPRIN induced IL-18 expression (Fig. 3F). The inhibition of IL-18 expression by the mutant expression vectors was not a non-specific cell death effect, since compared to a death-inducing control (Doxorubicin), none induced significant mono- and oligonucleosomes in the cytoplasmic fraction (Fig. 3G).

3.4. EMMPRIN activates NF- κ B and AP-1 via Rac1

Rac1 is a member of the rho family of small GTPases that plays a significant role in cell growth, migration, and transformation *in vitro*, and myocardial hypertrophy and failure *in vivo* [30,31]. It is also known to be involved in oxidative stress, and the activation of stress-responsive transcription factors such as NF- κ B [31]. Since EMMPRIN induced NF- κ B and AP-1 activations (Fig. 2), we hypothesized that this response is mediated via Rac1. EMMPRIN markedly activated Rac1 in adult ACM, an effect that was significantly attenuated by the forced expression of Myc-tagged dominant negative Rac1 ([MOI 50], Fig. 4A; expression of Myc is shown in the middle panel). Similar results were obtained when the Rac1 inhibitor NSC23766 was used (data not shown). Further, forced expression of N17rac1 significantly attenuated EMMPRIN-mediated IL-18 expression (Fig. 4B). Further investigations revealed that ectopic expression of N17rac1 inhibited nuclear p65 and cJun levels (Fig. 4C, immunoblotting; Fig. 4D, ELISA). Forced expression of N17rac1 also inhibited activation of JNK (Fig. 4E), as did the pharmacological inhibitor SP 600125 (Fig. 4F). Moreover, N17rac1 expression blunted EMMPRIN-mediated IKK β activity (Fig. 4G).

3.5. EMMPRIN activates cJun via MKK7

Since MKK7 is a specific activator of JNK [32], we next investigated whether MKK7 is downstream of Rac1. Indeed, results in Fig. 5A show that EMMPRIN is a potent inducer of MKK7 activity, an effect that was significantly inhibited by the forced expression of N17rac1. Further, knockdown of MKK7 inhibited EMMPRIN mediated JNK phosphorylation (Fig. 5B; knockdown of MKK7 was confirmed by immunoblotting, Fig. 5C) and cJun nuclear translocation (Fig. 5D). Similarly, N17rac1 expression or treatment with SP600125 also inhibited EMMPRIN-mediated cJun activation (Fig. 5D).

3.6. EMMPRIN activates IKK, but not JNK, via PI3K and Akt

Similar to its effects in aortic smooth muscle cells [10], EMMPRIN induced PI3K (Fig. 6A) and Akt (Fig. 6B) activities in ACM, effects that were significantly attenuated by the forced expression of N17rac1. Further, the PI3K inhibitor wortmannin (Fig. 6C) and the Akt inhibitor Akti-X (Fig. 6D) both attenuated EMMPRIN-induced Akt activation. Moreover, while inhibiting EMMPRIN-mediated IKK activity (Fig. 6E), wortmannin failed to significantly modulate JNK activation (Fig. 6F). Importantly, wortmannin and Akti-X both inhibited EMMPRIN-induced IL-18 mRNA expression, demonstrating that EMMPRIN induces IL-18 expression via PI3K-dependent Akt activation. This was further supported by the overexpression of constitutively active Akt (myr-Akt) in ACM, which induced IL-18 expression (Fig. 6H).

3.7. EMMPRIN upregulates MMPs and TIMPs in ACM in a time-dependent manner

To investigate the global effect of EMMPRIN on the MMP and TIMP families of genes, ACM were treated with EMMPRIN, and the RNA isolated at 1 h and 24 h for analysis using a qPCR-based array (PAMM-013; SABiosciences, Frederick, MD 21703, USA). Compared to untreated controls, EMMPRIN treatment for 1 h upregulated the expression of MMP2, 8, 9, 13 and 14 by 2–3-fold (Fig 7A). However, MMPs 1, 10, 11 15 and 16 were either extremely low or undetected. In addition, although TIMPS 1 and 3 were detected at basal conditions, their expression levels were not significantly modulated by EMMPRIN treatment. Of note, TIMP2 was not detected, and TIMP4 was not present on the array. However, the stimulatory effects of EMMPRIN were more pronounced at 24 h, with a 10–20-fold induction in MMPs 2, 8, 9, 13 and 14. Interestingly, at this time point, EMMPRIN also upregulated TIMPs 1 and 3 mRNA expressions. Using RT-qPCR, we further confirmed the time-dependent increases in MMP2 and 9 expressions (Fig. 7B).

4. DISCUSSION

As its name implies, EMMPRIN induces the expression of MMPs in a variety of cell systems, and modulates both physiological and pathological tissue remodeling. The proinflammatory cytokine IL-18 also modulates myocardial remodeling by inducing MMP expression and ECM degradation. EMMPRIN can also induce proinflammatory responses, for instance, by inducing the expression of several cytokines in monocytes [9] with resulting negative myocardial ionotropic effects. In the present study, we clearly demonstrate that EMMPRIN is a potent inducer of IL-18 expression in cardiomyocytes, stimulating *IL18* gene transcription and mRNA levels, and IL-18 protein expression and secretion. EMMPRIN activated NF- κ B, AP-1, CREB, and ATF-2 in cardiomyocytes, and induced IL-18 expression via Rac1-mediated PI3K/Akt/IKK-dependent I κ B- α degradation and NF- κ B activation, and by MKK7/JNK-dependent AP-1 activation (Fig. 7C). These results suggest that EMMPRIN and IL-18 together may form a mutually reinforcing response mechanism to myocardial injury that can result in adverse myocardial remodeling.

A critical observation in the present study is that EMMPRIN activates several transcription factors in cardiomyocytes, including NF- κ B (p50 and p65), AP-1 (cFos, cJun and FosB), CREB and ATF-2, suggesting that EMMPRIN may exert diverse biological effects in cardiomyocytes under normal and pathological conditions. Importantly, our results demonstrate that EMMPRIN stimulates IL-18 expression in cardiomyocytes primarily by inducing NF- κ B and AP-1 binding to the *IL18* promoter *in vivo*, and thus enhancing its transcription. Although EMMPRIN also stimulated the activation of CREB and ATF-2, the forced expression of KCREB failed to modulate EMMPRIN-induced IL-18 expression suggesting a minor role if any, in the direct regulation of IL-18. However, it is possible that EMMPRIN-induced CREB may be indirectly involved by regulating its own induction, and

that of cFos and JunD [33], thus amplifying AP-1-dependent signaling. By collaborating with members of AP-1 and NF- κ B family of proteins, CREB might also induce other proinflammatory cytokines in cardiomyocytes, including IL-6 and TNF- α . For example, cooperation between NF- κ B and CREB has been shown to induce IL-6 expression in astrocytes through enhanced recruitment of the limiting cofactor CREB binding protein or CBP [34]. Our results also demonstrate that EMMPRIN activates ATF-2 (CREB2) in cardiomyocytes. ATF-2 homodimers or ATF-2/Jun heterodimers can bind CRE or TPA-responsive elements in the promoter region of target genes and regulate their expression [35]. EMMPRIN induced the phosphorylation of ATF-2 on Thr69/Thr71, implying that EMMPRIN may activate various SAPK/MAPKs in cardiomyocytes. In fact, our results showed that EMMPRIN mediates JNK activation. Of note, ATF-2 is also a target of JNK signal transduction pathway [36]. Studies are in progress to determine whether EMMPRIN also activates p38 MAPK and ERK1/2 in cardiomyocytes, and examine their role in EMMPRIN-dependent gene expression. It should be noted that in the TF arrays studies, we only examined the effect of EMMPRIN on cardiomyocytes after 2 h of incubation, which is perhaps a limitation since transcription factors are regulated both differentially and temporally. TF array studies are currently in progress to determine the temporal expression and regulation of TFs in cardiomyocytes in response to EMMPRIN.

We demonstrated that EMMPRIN activates NF- κ B and AP-1 in a Rac1-dependent manner. Rac1 is a small GTPase that functions as a regulatory subunit in some NADPH oxidases [37]. As such, it may be involved in the extra-mitochondrial generation of ROS and oxidative stress. It will be important to determine whether EMMPRIN induces oxidative stress in cardiomyocytes, and whether this oxidative stress *per se* contributes to gene induction and adverse myocardial remodeling. Our results also show that EMMPRIN activates NF- κ B in PI3K-dependent manner. Although PI3K can function upstream or downstream of Rac1, depending on cell type and stimulus, we found that forced expression of N17rac1 inhibited PI3K activation, thus suggesting that EMMPRIN activates NF- κ B and IL-18 expression via Rac1-dependent PI3K activation. Of note, activation of PI3K has been shown to promote translocation of Rac1 from cytosol to the plasma membrane [37], where it interacts with NADPH oxidase and generates ROS.

EMMPRIN also induced IKK activation in a PI3K/Akt-dependent manner. IKK in turn induced I κ B- α phosphorylation and degradation, demonstrating that EMMPRIN activates NF- κ B via a Rac1/PI3K/Akt/IKK signaling pathway. In contrast, inhibition of PI3K failed to significantly modulate JNK activation. However, forced expression of N17rac1 attenuated JNK activation, suggesting that EMMPRIN activates JNK via Rac1, but not via PI3K. Further, our results also demonstrate that EMMPRIN activates JNK-dependent cJun activation via MKK7. Though a similar signaling pathway was reported previously in H-ras-induced fibroblast cell death [38], EMMPRIN failed to significantly modulate cardiomyocyte viability (data not shown), suggesting that EMMPRIN is not pro-apoptotic in cardiomyocytes.

Another critical observation in the present study is that EMMPRIN upregulates several MMPs and their inhibitors in cardiomyocytes. In addition to its well-described stimulatory effects on MMP2 and 14 transcription, our results also show a moderate 2–3 fold induction of MMPs 3, 7, 8, and 13 at 1 h following EMMPRIN addition, but a robust 10–20-fold induction after 24 h. Considering that EMMPRIN activates multiple transcription factors, including AP-1 and NF- κ B, it is not entirely surprising that EMMPRIN induces various MMPs *in vitro* in cardiomyocytes. For example, MMPs 3, 7, 9, 10, 12 and 13 contain at least one AP-1 binding site in their proximal promoter region [39]. MMPs 2, 3, 9 and 14 are also NF- κ B responsive in a cell and stimulus-specific manner. In fact, we have previously demonstrated that both AP-1 and NF- κ B are critical for MMP9 transcription in IL-18-treated

aortic smooth muscle cells[40]. Since EMMPRIN activates several transcription factor elements, it is also possible that the crosstalk between these and other *cis* elements can upregulate MMPs expression. It is also possible that their robust expression at 24 h may be secondary to the induction of other factors induced by EMMPRIN (*e.g.*, IL-18 and other cytokines). Studies are in progress to determine whether increased MMP transcription translates to enhanced protein synthesis, secretion and activation. Interestingly, our results also show that EMMPRIN induces TIMPs 1 and 3 expressions in a delayed fashion. Of note, both TIMPs 1 and 3 are also AP-1 responsive [41,42]. Although TIMPs are potent inhibitors of MMPs, expression levels of several MMPs are significantly upregulated at 24 h, suggesting that EMMPRIN upregulation favors unopposed MMP expression, ECM destruction, and adverse remodeling.

An interesting and potentially important observation of the present study is that EMMPRIN induces or activates several factors involved in cell survival or growth. For example, (i) EMMPRIN activates Rac1 GTPase in cardiomyocytes. Rac1 overexpression results in cardiomyocyte growth *in vitro* and myocardial hypertrophy *in vivo* [31]. In contrast, its deletion in a cardiomyocyte-specific manner attenuates angiotensin-II induced myocardial hypertrophy [31]. (ii) EMMPRIN activates MKK7, a pro-hypertrophic factor that induces cardiomyocyte growth via JNK [43]. (iii) EMMPRIN activates the well-known pro-survival and pro-growth factors PI3K and Akt. (iv) EMMPRIN induces NF- κ B activation. Activation of NF- κ B plays a role in both cell survival and apoptosis. However, our results show that EMMPRIN fails to induce cardiomyocyte death. (v) EMMPRIN induces ATF-2 activation, and ATF-2 mediates TGF β 1-induced cardiac hypertrophy [44]. Of note, MMPs proteolytically activate TGF- β from extracellular matrix [45]. (vi) EMMPRIN induces IL-18 expression. We and others have previously demonstrated that IL-18 is a potent pro-hypertrophic and pro-growth factor both *in vitro* and *in vivo* [19,46]. (vii) EMMPRIN also induces the expression of IL-1 β , IL-6, and TNF- α [9]. These cytokines also play a role in myocardial hypertrophy. Importantly, (viii) EMMPRIN overexpression in cardiomyocyte-specific manner induces myocardial hypertrophy and failure *in vivo* [14]. Since EMMPRIN activates several factors involved in cell growth as discussed above and its overexpression induces myocardial hypertrophy, we hypothesize that EMMPRIN is a pro-survival and pro-hypertrophic agent. Studies are in progress to examine comprehensively the gene expression and signal transduction pathways regulated by EMMPRIN, and characterize its cell growth effects, if any, in cardiomyocytes using exogenous addition of recombinant EMMPRIN and by the adenoviral transduction of wild-type EMMPRIN. Our unpublished studies also demonstrate that curcumin, an active ingredient of *Curcumin longa*, inhibits EMMPRIN signaling and IL-18 expression, suggesting its therapeutic potential in adverse remodeling. Since remodeling, hypertrophy and failure are complex disease processes [47,48], supplementing therapeutic regimens with curcumin may further improve the outcome by inhibiting EMMPRIN/IL-18 signaling and adverse remodeling.

In summary, our results demonstrate that EMMPRIN activates NF- κ B, AP-1, CREB, and ATF-2 in cardiomyocytes, induces IL-18 expression via Rac1-dependent PI3K/Akt/IKK/NF- κ B and MKK7/JNK/AP-1 activations. Further, EMMPRIN regulates expression of both MMPs and TIMPs in a time-dependent manner. These results suggest that by regulating genes involved in inflammation, injury, and ECM destruction, EMMPRIN plays a causal role in adverse remodeling, and thus a potential therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the NIH-NHLBI grant HL-86787 (BC), HL-70241 and HL-80682 (PD), and by award number 1101BX007080 from the Biomedical Laboratory Research and Development Service of the VA Office of Research and Development (BC). "The contents of this report do not represent the views of the Department of Veterans Affairs or the United States Government."

References

1. Li YY, McTiernan CF, Feldman AM. Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling. *Cardiovasc Res* 2000 May; 46(2):214–24. [PubMed: 10773225]
2. Huet E, Gabison EE, Mourah S, Menashi S. Role of emmprin/CD147 in tissue remodeling. *Connect Tissue Res* 2008;49(3):175–9. [PubMed: 18661337]
3. Iacono KT, Brown AL, Greene MI, Saouaf SJ. CD147 immunoglobulin superfamily receptor function and role in pathology. *Exp Mol Pathol* 2007 Dec;83(3):283–95. [PubMed: 17945211]
4. Sun J, Hemler ME. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. *Cancer Res* 2001 Mar 1;61(5):2276–81. [PubMed: 11280798]
5. Tang Y, Kesavan P, Nakada MT, Yan L. Tumor-stroma interaction: positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. *Mol Cancer Res* 2004 Feb;2(2):73–80. [PubMed: 14985463]
6. Igakura T, Kadomatsu K, Kaname T, Muramatsu H, Fan QW, Miyauchi T, et al. A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. *Dev Biol* 1998 Feb 15;194(2):152–65. [PubMed: 9501026]
7. Kuno N, Kadomatsu K, Fan QW, Hagihara M, Senda T, Mizutani S, et al. Female sterility in mice lacking the basigin gene, which encodes a transmembrane glycoprotein belonging to the immunoglobulin superfamily. *FEBS Lett* 1998 Mar 27;425(2):191–4. [PubMed: 9559645]
8. Schmidt R, Bultmann A, Ungerer M, Joghetaei N, Bulbul O, Thieme S, et al. Extracellular matrix metalloproteinase inducer regulates matrix metalloproteinase activity in cardiovascular cells: implications in acute myocardial infarction. *Circulation* 2006 Feb 14;113(6):834–41. [PubMed: 16461815]
9. Schmidt R, Bultmann A, Fischel S, Gillitzer A, Cullen P, Walch A, et al. Extracellular matrix metalloproteinase inducer (CD147) is a novel receptor on platelets, activates platelets, and augments nuclear factor kappaB-dependent inflammation in monocytes. *Circ Res* 2008 Feb 15;102(3):302–9. [PubMed: 18048771]
10. Venkatesan B, Valente AJ, Reddy VS, Siwik DA, Chandrasekar B. Resveratrol blocks interleukin-18-EMMPRIN cross-regulation and smooth muscle cell migration. *Am J Physiol Heart Circ Physiol* 2009 Aug;297(2):H874–86. [PubMed: 19561311]
11. Siwik DA, Kuster GM, Brahmabhatt JV, Zaidi Z, Malik J, Ooi H, et al. EMMPRIN mediates beta-adrenergic receptor-stimulated matrix metalloproteinase activity in cardiac myocytes. *J Mol Cell Cardiol* 2008 Jan;44(1):210–7. [PubMed: 17869266]
12. Fielitz J, Leuschner M, Zurbrugg HR, Hannack B, Pregla R, Hetzer R, et al. Regulation of matrix metalloproteinases and their inhibitors in the left ventricular myocardium of patients with aortic stenosis. *J Mol Med* 2004 Dec;82(12):809–20. [PubMed: 15558223]
13. Spinale FG, Coker ML, Heung LJ, Bond BR, Gunasinghe HR, Etoh T, et al. A matrix metalloproteinase induction/activation system exists in the human left ventricular myocardium and is upregulated in heart failure. *Circulation* 2000 Oct 17;102(16):1944–9. [PubMed: 11034943]
14. Zavadzka JA, Plyler RA, Bouges S, Koval CN, Rivers WT, Beck CU, et al. Cardiac-restricted overexpression of extracellular matrix metalloproteinase inducer causes myocardial remodeling and dysfunction in aging mice. *Am J Physiol Heart Circ Physiol* 2008 Oct;295(4):H1394–402. [PubMed: 18689500]

15. Dinarello CA, Novick D, Puren AJ, Fantuzzi G, Shapiro L, Muhl H, et al. Overview of interleukin-18: more than an interferon-gamma inducing factor. *J Leukoc Biol* 1998 Jun;63(6): 658–64. [PubMed: 9620656]
16. Zabalgoitia M, Colston JT, Reddy SV, Holt JW, Regan RF, Stec DE, et al. Carbon monoxide donors or heme oxygenase-1 (HO-1) overexpression blocks interleukin-18-mediated NF-kappaB-PTEN-dependent human cardiac endothelial cell death. *Free Radic Biol Med* 2008 Feb 1;44(3): 284–98. [PubMed: 18215737]
17. Chandrasekar B, Mummidi S, Valente AJ, Patel DN, Bailey SR, Freeman GL, et al. The pro-atherogenic cytokine interleukin-18 induces CXCL16 expression in rat aortic smooth muscle cells via MyD88, interleukin-1 receptor-associated kinase, tumor necrosis factor receptor-associated factor 6, c-Src, phosphatidylinositol 3-kinase, Akt, c-Jun N-terminal kinase, and activator protein-1 signaling. *J Biol Chem* 2005 Jul 15;280(28):26263–77. [PubMed: 15890643]
18. Yu Q, Vazquez R, Khojini EV, Patel C, Venkataramani R, Larson DF. IL-18 induction of osteopontin mediates cardiac fibrosis and diastolic dysfunction in mice. *Am J Physiol Heart Circ Physiol* 2009 Jul;297(1):H76–85. [PubMed: 19429811]
19. Chandrasekar B, Mummidi S, Claycomb WC, Mestril R, Nemer M. Interleukin-18 is a pro-hypertrophic cytokine that acts through a phosphatidylinositol 3-kinase-phosphoinositide-dependent kinase-1-Akt-GATA4 signaling pathway in cardiomyocytes. *J Biol Chem* 2005 Feb 11;280(6):4553–67. [PubMed: 15574430]
20. Venkatachalam K, Prabhu SD, Reddy VS, Boylston WH, Valente AJ, Chandrasekar B. Neutralization of interleukin-18 ameliorates ischemia/reperfusion-induced myocardial injury. *J Biol Chem* 2009 Mar 20;284(12):7853–65. [PubMed: 19164288]
21. Colston JT, Boylston WH, Feldman MD, Jenkinson CP, de la Rosa SD, Barton A, et al. Interleukin-18 knockout mice display maladaptive cardiac hypertrophy in response to pressure overload. *Biochem Biophys Res Commun* 2007 Mar 9;354(2):552–8. [PubMed: 17250807]
22. Wang M, Tan J, Wang Y, Meldrum KK, Dinarello CA, Meldrum DR. IL-18 binding protein-expressing mesenchymal stem cells improve myocardial protection after ischemia or infarction. *Proc Natl Acad Sci U S A* 2009 Oct 13;106(41):17499–504. [PubMed: 19805173]
23. Mallat Z, Heymes C, Corbaz A, Logeart D, Alouani S, Cohen-Solal A, et al. Evidence for altered interleukin 18 (IL)-18 pathway in human heart failure. *FASEB J* 2004 Nov;18(14):1752–4. [PubMed: 15371332]
24. Venkatesan B, Prabhu SD, Venkatachalam K, Mummidi S, Valente AJ, Clark RA, et al. WNT1-inducible signaling pathway protein-1 activates diverse cell survival pathways and blocks doxorubicin-induced cardiomyocyte death. *Cell Signal* May;22(5):809–20. [PubMed: 20074638]
25. Imam SZ, Jankovic J, Ali SF, Skinner JT, Xie W, Conneely OM, et al. Nitric oxide mediates increased susceptibility to dopaminergic damage in Nurr1 heterozygous mice. *FASEB J* 2005 Sep; 19(11):1441–50. [PubMed: 16126911]
26. Benotmane AM, Hoylaerts MF, Collen D, Belayew A. Nonisotopic quantitative analysis of protein-DNA interactions at equilibrium. *Anal Biochem* 1997 Aug 1;250(2):181–5. [PubMed: 9245437]
27. Chandrasekar B, Marelli-Berg FM, Tone M, Bysani S, Prabhu SD, Murray DR. Beta-adrenergic stimulation induces interleukin-18 expression via beta2-AR, PI3K, Akt, IKK, and NF-kappaB. *Biochem Biophys Res Commun* 2004 Jun 25;319(2):304–11. [PubMed: 15178407]
28. Tone M, Thompson SA, Tone Y, Fairchild PJ, Waldmann H. Regulation of IL-18 (IFN-gamma-inducing factor) gene expression. *J Immunol* 1997 Dec 15;159(12):6156–63. [PubMed: 9550417]
29. Mollen KP, McCloskey CA, Tanaka H, Prince JM, Levy RM, Zuckerbraun BS, et al. Hypoxia activates c-Jun N-terminal kinase via Rac1-dependent reactive oxygen species production in hepatocytes. *Shock* 2007 Sep;28(3):270–7. [PubMed: 17545941]
30. Sawada N, Li Y, Liao JK. Novel aspects of the roles of Rac1 GTPase in the cardiovascular system. *Curr Opin Pharmacol* Apr;10(2):116–21. [PubMed: 20060361]
31. Satoh M, Ogita H, Takeshita K, Mukai Y, Kwiatkowski DJ, Liao JK. Requirement of Rac1 in the development of cardiac hypertrophy. *Proc Natl Acad Sci U S A* 2006 May 9;103(19):7432–7. [PubMed: 16651530]

32. Tournier C, Dong C, Turner TK, Jones SN, Flavell RA, Davis RJ. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev* 2001 Jun 1;15(11):1419–26. [PubMed: 11390361]
33. Mayr B, Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2001 Aug;2(8):599–609. [PubMed: 11483993]
34. Spooren A, Kooijman R, Lintermans B, Van Craenenbroeck K, Vermeulen L, Haegeman G, et al. Cooperation of NFkappaB and CREB to induce synergistic IL-6 expression in astrocytes. *Cell Signal* May;22(5):871–81. [PubMed: 20100571]
35. van Dam H, Castellazzi M. Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. *Oncogene* 2001 Apr 30;20(19):2453–64. [PubMed: 11402340]
36. Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* 1996 Oct;74(10):589–607. [PubMed: 8912180]
37. Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* 2006 Mar 3;98(4):453–62. [PubMed: 16514078]
38. Byun JY, Yoon CH, An S, Park IC, Kang CM, Kim MJ, et al. The Rac1/MKK7/JNK pathway signals upregulation of Atg5 and subsequent autophagic cell death in response to oncogenic Ras. *Carcinogenesis* 2009 Nov;30(11):1880–8. [PubMed: 19783847]
39. Yan C, Boyd DD. Regulation of matrix metalloproteinase gene expression. *J Cell Physiol* 2007 Apr;211(1):19–26. [PubMed: 17167774]
40. Chandrasekar B, Mummidi S, Mahimainathan L, Patel DN, Bailey SR, Imam SZ, et al. Interleukin-18-induced human coronary artery smooth muscle cell migration is dependent on NF-kappaB-and AP-1-mediated matrix metalloproteinase-9 expression and is inhibited by atorvastatin. *J Biol Chem* 2006 Jun 2;281(22):15099–109. [PubMed: 16554298]
41. Kim H, Pennie WD, Sun Y, Colburn NH. Differential functional significance of AP-1 binding sites in the promoter of the gene encoding mouse tissue inhibitor of metalloproteinases-3. *Biochem J* 1997 Jun 1;324(Pt 2):547–53. [PubMed: 9182717]
42. Clark IM, Rowan AD, Edwards DR, Bech-Hansen T, Mann DA, Bahr MJ, et al. Transcriptional activity of the human tissue inhibitor of metalloproteinases 1 (TIMP-1) gene in fibroblasts involves elements in the promoter, exon 1 and intron 1. *Biochem J* 1997 Jun 1;324(Pt 2):611–7. [PubMed: 9182725]
43. Wang Y, Su B, Sah VP, Brown JH, Han J, Chien KR. Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells. *J Biol Chem* 1998 Mar 6;273(10):5423–6. [PubMed: 9488659]
44. Lim JY, Park SJ, Hwang HY, Park EJ, Nam JH, Kim J, et al. TGF-beta1 induces cardiac hypertrophic responses via PKC-dependent ATF-2 activation. *J Mol Cell Cardiol* 2005 Oct;39(4):627–36. [PubMed: 16125722]
45. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000 Jan 15;14(2):163–76. [PubMed: 10652271]
46. Woldbaek PR, Sande JB, Stromme TA, Lunde PK, Djurovic S, Lyberg T, et al. Daily administration of interleukin-18 causes myocardial dysfunction in healthy mice. *Am J Physiol Heart Circ Physiol* 2005 Aug;289(2):H708–14. [PubMed: 15821032]
47. Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, Schulz R. The myocardial JAK/STAT pathway: from protection to failure. *Pharmacol Ther* 2008 Nov;120(2):172–85. [PubMed: 18786563]
48. Heusch G, Boengler K, Schulz R. Cardioprotection: nitric oxide, protein kinases, and mitochondria. *Circulation* 2008 Nov 4;118(19):1915–9. [PubMed: 18981312]

Fig. 1.

EMMPRIN induces IL-18 expression in ACM. RT-qPCR analysis of EMMPRIN-dependent IL-18 mRNA expression at 2 h in ACM. $*P < 0.05$, $**P < 0.001$ vs. untreated (n=6). (B) Specificity of EMMPRIN. ACM were incubated with anti-EMMPRIN neutralizing antibodies or infected with Ad.mEMMPRIN prior to EMMPRIN (1 ng/ml for 2 h) addition. $*P < 0.001$ vs. untreated, $\dagger P < 0.05$ vs. EMMPRIN (n=6). (C) EMMPRIN-induced IL-18 mRNA expression in ACM was confirmed by Northern blotting. (D) EMMPRIN induces IL-18 protein expression in ACM (n=3). (E) EMMPRIN stimulates IL-18 secretion. ACM were treated with EMMPRIN for 24 h, and secreted IL-18 was quantified by ELISA. $*P < 0.001$ vs. untreated (n=6). (F) The contaminating low levels of endotoxin in EMMPRIN are not responsible for EMMPRIN-mediated IL-18 expression. ACM incubated with polymyxin B prior to EMMPRIN addition were analyzed for IL-18 expression at 8 h by immunoblotting (n=3).

Fig. 2.

Parallel profiling of transcription factors (TF) regulated by EMMPRIN in ACM. (A) EMMPRIN activates multiple transcription factors in ACM. ACM treated with EMMPRIN (1 ng/ml for 2 h) were analyzed for protein-DNA interactions in pooled nuclear extracts using TransSignal Protein/DNA Array 1. Purity and equal loading of nuclear proteins extracts were confirmed by immunoblotting (right hand panel) using antibodies against Lamin A/C (nuclear) and GAPDH (cytoplasmic). (B) EMMPRIN activated NF- κ B, AP1, and CREB in ACM nuclear extracts were analyzed by an ELISA. $P < 0.05$ vs. control (n=3/group). (C) EMMPRIN-mediated NF- κ B activation in ACM was confirmed by a reporter assay. $*P < 0.001$ vs. Ad. MCS-Luc+EMMPRIIN (n=12). (D) EMMPRIN promotes p65 nuclear translocation in ACM (n=3). (E) EMMPRIN-stimulated NF- κ B binding to *IL18* promoter *in vivo* was confirmed by the ChIP assay (n=3). (F) EMMPRIN stimulates AP-1-dependent reporter gene activation. $*P < 0.001$ vs. Ad. MCS-Luc+EMMPRIIN (n=12). (G) EMMPRIN-stimulated AP-1 binding to *IL18* promoter *in vivo* was confirmed by the ChIP assay (n=3). (H) EMMPRIN activates CREB. ACM treated with EMMPRIN were analyzed for phospho-CREB (Ser133) levels in nuclear protein extracts by immunoblotting. Total CREB served as a loading control (n=3). (I) EMMPRIN activates ATF-2. ACM treated as in 2D, but for up to 3 h, were analyzed for phospho-ATF-1 (Thr69/Thr71) levels in nuclear protein extracts by immunoblotting. Total ATF-2 served as a loading control (n=3).

Fig. 3.

EMMPRIN induces IL-18 expression via NF- κ B and AP-1. (A) EMMPRIN stimulates IL18 promoter-dependent reporter gene activation in NMCM via NF- κ B and AP-1, but not CREB. $*P < 0.001$ vs. untreated, $\dagger P < \text{at least } 0.05$ vs. EMMPRIN ($n=12$). (B) EMMPRIN induces IL-18 mRNA expression via NF- κ B and AP-1. ACM infected with adenoviral mutant NF- κ B or CREB vectors for 24 h were incubated with EMMPRIN addition, and the analyzed for IL-18 mRNA expression by RT-qPCR. $*P < 0.001$ vs. untreated, $\dagger P < \text{at least } 0.05$ vs. EMMPRIN ($n=12$). (C) EMMPRIN induces IL-18 protein expression via NF- κ B. ACM treated with EMMPRIN were analyzed for IL-18 protein expression at 8 h by immunoblotting ($n=3$). (D) EMMPRIN induces IL-18 protein expression via AP-1. ACM infected with Ad.dncJun prior to EMMPRIN addition. IL-18 protein expression was analyzed at 8 h by immunoblotting ($n=3$). (E) Ectopic expression of KCREB inhibits EMMPRIN-mediated CREB activation. ACM infected with Ad. KCREB prior to EMMPRIN addition were analyzed for total and pCREB levels at 1 h by immunoblotting ($n=3$). (F) Ectopic expression of KCREB fails to modulate EMMPRIN-mediated IL-18 expression ($n=3$). (G) Adenoviral transduction of mutant NF- κ B, AP-1 and CREB fail to induce cell death. ACM infected with the indicated adenoviral vectors for 24 h were analyzed for cell death by quantifying mono and oligonucleosomal fragmented DNA in the cytoplasmic extracts. Doxorubicin served as a positive control. $*P < 0.001$ vs. untreated ($n=12$).

Fig. 4.

EMMPRIN activates NF- κ B and AP-1 via Rac1. (A) EMMPRIN-stimulated Rac1 activity in ACM at 30 min is blunted by Ad. N17rac1 (n=3). (B) EMMPRIN induces IL-18 expression via Rac1. ACM infected with Ad. N17rac1 prior to EMMPRIN addition were analyzed for IL-18 protein expression by immunoblotting at 8 h (n=3). (C), (D) EMMPRIN stimulates p55 and c-Jun nuclear translocation via Rac1. ACM infected with Ad. N17rac1 prior to EMMPRIN addition were analyzed for p55 and c-Jun in nuclear extracts by immunoblotting (C) and ELISA (D), * $P < 0.01$ vs. untreated, † $P < 0.05$ vs. EMMPRIN (n=3). (E) EMMPRIN induces JNK activation via Rac1. ACM treated with EMMPRIN for 1 h were analyzed for total and phospho-JNK (Thr183/Tyr185) levels by immunoblotting (n=3). (F) SP 600125 inhibits EMMPRIN-mediated JNK activation in ACM (n=3). (G) EMMPRIN stimulates IKK β activity via Rac1. ACM infected with Ad. N17rac1 prior to EMMPRIN addition were analyzed for IKK β activity at 30 min by an *in vitro* kinase assay (n=3).

Fig. 5.

EMMPRIN activates cJun via MKK7. (A) EMMPRIN induces MKK7 activation via Rac1. ACM were infected with Ad. N17rac1 prior to EMMPRIN addition, and then analyzed for MKK7 activity using immune complex kinase assays (n=3). (B) EMMPRIN stimulates JNK activation via MKK7. ACM were infected with lentiviral MKK7 shRNA for 24 h prior to EMMPRIN addition, and then analyzed for JNK activity at 1 h by immunoblotting (n=3). (C) Knockdown of MKK7 was confirmed by immunoblotting (n=3). (D) EMMPRIN induces cJun activation via Rac1, MKK7 and JNK. ACM were either infected with N17rac1 or MKK7 shRNA, or treated with SP600125 prior to EMMPRIN addition. cJun levels in nuclear protein extracts at 2 h is analyzed by ELISA. * $P < 0.01$ vs. untreated, † $P < 0.05$ vs. EMMPRIN (n=3).

Fig. 6.

EMMPRIN activates NF- κ B and AP-1 via Rac1-dependent PI3K/Akt signaling. (A) EMMPRIN activates PI3K via Rac1. ACM were infected with Ad. N17rac1 prior to EMMPRIN addition, and then analyzed for PI3K activation at 1 h by ELISA. top: immunoblot analysis of the same samples with anti-p85 antibody. * $P < 0.01$ vs. untreated; † $P < 0.001$ vs. EMMPRIN (n = 6). (B) EMMPRIN activates Akt via Rac1. ACM treated as in A were analyzed for Akt activation by immunoblotting (n=3). (C) Wortmannin inhibits EMMPRIN-mediated Akt activation in ACM (n=3). (D) Akti-X inhibits EMMPRIN-mediated Akt activation in ACM (n=3). (E) Wortmannin inhibits EMMPRIN-induced IKK activity in ACM at 30 min (n=3). (F) Wortmannin fails to inhibit EMMPRIN-induced JNK activation in ACM (n=3). (G) Wortmannin and Akti-X inhibit EMMPRIN-induced IL-18 mRNA expression in ACM at 2 h as analyzed by RT-qPCR. * $P < 0.001$ vs. untreated; † $P < 0.01$ vs. EMMPRIN (n = 6). (H) Forced expression of constitutively active Akt stimulates IL-18 expression. ACM infected with HA-tagged constitutively active Akt (Ad.myr-Akt) for 24 h were analyzed for IL-18 mRNA expression by RT-qPCR. * $P < 0.001$ vs. untreated or Ad. GFP (n = 6).

Fig. 7.

EMMPRIN induces various matrix metalloproteinases and their tissue inhibitors in ACM.

(A) ACM treated with EMMPRIN for 1 and 24 h were analyzed for MMPs and TIMPs using a qPCR-based array. $*P < 0.05$ vs control (n=6). (B) EMMPRIN-induced MMP2 and MMP9 expressions were confirmed by RT-qPCR. $*P < 0.05$, $**P < 0.001$ vs untreated (n=6). (C) Schema showing possible signal transduction pathway involved in EMMPRIN signaling. While the pathways highlighted by blue are proven experimentally, the signal transduction pathways highlighted in red are yet to be investigated.

TABLE 1

Primers used in the Protein/DNA array 1

Transcription factor	Lanes on array	DNA sequence
AP-1 (1)	A1, A2	CGCTTGATGACTCAGCCGGAA
AP-1 (2)	A17, A18	GATCAGCTTGATGATGAGTCAGCCCG
AP-2 (1)	A3, A4	GATCGAACTGACCGCCCGCGGCCCGT
CREB	C1, C2	AGAGATTGCCTGACGTCAGAGAGCTAG
NF- κ B	G7, G8	AGTTGAGGGGACTTCCCAGGC