Published in final edited form as:

Biochem Biophys Res Commun. 2011 December 2; 415(4): 662–668. doi:10.1016/j.bbrc.2011.10.132.

Regulation of MMP10 Expression by the Transcription Factor CHF1/Hey2 is Mediated by Multiple E Boxes

Ling Wu, Wei-Ming Chien, Matthew E. Hartman, Farid Moussavi-Harami, Yonggang Liu, and Michael T. Chin*

Division of Cardiology, Department of Medicine, University of Washington, Seattle, WA

Abstract

The cardiovascular restricted bHLH transcription factor CHF1/Hey2 has been reported to play an important role in regulation of vascular smooth muscle phenotype and gene expression, but the downstream target genes that mediate these effects have not been completely elucidated. We have previously found that loss of CHF1/Hey2 in vascular smooth muscle cells leads to dysregulated expression of the matrix metalloproteinase *MMP10* after treatment with PDGF. Here we report that loss or knockdown of CHF1/Hey2 in vascular smooth muscle cells leads to increased expression and activity of *MMP10* at baseline, suggesting a direct effect of CHF1/Hey2 on *MMP10* promoter regulation. To test this hypothesis, we assessed the effects of CHF1/Hey2 on a 2.5kb *MMP10* promoter region upstream of the transcriptional start site. We found that this region contains multiple elements including 12 E-boxes that mediate constitutive activity and repression by CHF1/Hey2 in 293T cells and A7r5 smooth muscle cells. Surprisingly, mutation of these E-boxes not only abolished CHF1/Hey2 repression, but also diminished constitutive expression. In addition, we observed that some of these mutations unmasked an activator function for CHF1/Hey2, which has not been previously described. These findings support the hypothesis that CHF1/Hey2 is an important regulator of *MMP10* expression.

Keywords

transcription factor; Hey2; MMP10; matrix metalloproteinase; vascular smooth muscle cells

Introduction

Cardiovascular basic helix-loop-helix factor 1 (CHF1, also referred to as Hey2, Hesr, Hrt, HERP and gridlock) belongs to a family of hairy-related transcription factors that play important roles in cardiovascular development and adult cardiovascular biology. CHF1/Hey2 is expressed in the ventricle and vasculature during embryonic stages and at lower levels after birth. The importance of CHF1/Hey2 in the development of the vascular system has been shown in zebrafish [1,2] and in mice [3]. A mutation in *gridlock*, which encodes the ortholog of CHF1/Hey2, selectively disrupts assembly of the aorta and causes coarctation at the aortic bifurcation. In mice, global deletion of CHF1/Hey2 leads to abnormal patterning and maturation of the coronary arteries, characterized by abnormal

© 2011 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed at: Center for Cardiovascular Biology, University of Washington School of Medicine, Box 358050, 815 Mercer Street, Room 353, Seattle, WA 98109, Tel. 206 616-8880, Fax: 206 616-4082, mtchin@u.washington.edu.

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.

smooth muscle development. CHF1/Hey2 has also been shown to function as a transcriptional repressor of myocardin-dependent smooth muscle marker genes, SM-MHC and SM22 α , through the disrupting the binding between SRF and the CArG boxes in the promoter region of these smooth muscle marker genes [4] or through some other mechanism that does not involve protein-protein interaction [5]. It has also been shown to repress Notch-dependent activation of the smooth muscle alpha actin promoter [6]. Knockout mice also show decreased neointimal formation after femoral arterial wire injury and decreased proliferation in CHF1/Hey2-deficient smooth muscle cells (SMCs) compared with wild-type SMCs [7]. The downstream targets of CHF1/Hey2 that regulate smooth muscle phenotype have not been completely elucidated.

Our previous study identifying potential target genes downstream of CHF1/Hey2 has demonstrated that matrix metalloproteinase-10 (MMP-10) was upregulated in SMCs isolated from CHF1/Hey2 knockout mice and treated with platelet-derived growth factor (PDGF) [8]. MMP-10 (also called stromelysin-2) belongs to the matrix metalloproteinase (MMP) family, which contains at least 28 zinc-dependent endopeptidases [9] that are divided into six groups: collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and MMP-26), membrane-type (MT)-MMPs (MMP-14, -15, -16, -17, -24 and -25) and others (MMP-12, -19, -20, -21, -23, -27 and -28). MMPs are found as secreted enzymes, cell surface enzymes and intracellular enzymes [10] and function in the degradation of collagen, elastin, and other bioactive components of the extracellular matrix (ECM). MMPs are initially synthesized as inactive zymogens (pro-MMPs) and proteolytically converted to the active enzymes. MMP-10 is known to play an important role in cell proliferation, migration, differentiation and angiogenesis, as well as vascular integrity [11]. Highly expressed MMP-10 has been founded in several human tumors of epithelial origin and several stages of the tumorigenic process [12].

To verify that CHF1/Hey2 regulates *MMP10* expression, we measured expression and enzyme activity in knockout cells and after siRNA knockdown. We also tested whether CHF1/Hey2 directly regulates the *MMP10* promoter and performed mutational analysis to identify important elements that mediate this effect. Here we report that knockdown of CHF1/Hey2 in SMCs directly leads to increased expression of *MMP10*, that CHF1/Hey2 directly represses the *MMP10* promoter in reporter assays and that its effects are mediated by multiple E boxes.

Materials and methods

Construction of Plasmids and Antibodies

The mouse *MMP10* upstream promoter fragments 2.5kb, 2kb, 1.5 kb, 1kb and 0.5kb upstream of exon1 were amplified by PCR from C57BL/6 mouse genomic DNA and cloned into the commercially available firefly luciferase reporter vector, pGL3-basic (Promega) at the XhoI and HindIII (underlined) sites, using the following primers: forward: 2.5kb: 5'aattcaaactcgagccttcattagaatcttctgtacc 3'; 2kb: 5'aattcaaactcgagtttctacatgccaccctgagtc 3'; 1.5kb: 5'aattcaaactcgagtcccctccagggaaattttgt 3'; 1kb: 5'aattcaaactcgagtatgagcatctcagaatgagtcg 3' and 0.5kb: 5'aattcaaactcgagtacctctgtacattcctcctccg 3'; reverse, 5'aattcaaaagcttctagcacctggttccatatttc 3'). The E-boxes (CANNTG) in the promoter were mutated using a QuikChange Site-Directed Mutagenesis Kit (Invitrogen). Primer sequences for each mutagenic oligonucleotide are available upon request. Site1, 3, 4 and site6 E-boxes are replaced with CGGTCG and the other 8 E-boxes were replaced with CGTTTCG. The CHF1/Hey2 expression vector pCDNA3-hCHF1 has been described previously [13]. The MMP-10 mouse monoclonal antibody was purchased from Millipore (MAB13422).

Transient transfection and luciferase reporter assays

293T and A7r5 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were plated onto six-well trays and transfected when 50–70% confluency with the indicated plasmids and an internal control plasmid (pcDNA3.1/Hygro/LacZ, Invitrogen) encoding beta-galactosidase, mixed with FuGENE HD transfection reagent according to the manufacturer's instructions (Roche). Cells were harvested with passive lysis buffer after 24 h and luciferase assays were performed according to the manufacturer's protocol (Promega). Beta-galactosidase activity was measured using a beta-galactosidase enzyme assay system (Promega) and was used to normalize the results for transfection efficiency. All experiments were repeated independently at least 3 times, and the results are shown as mean $\pm$ SD.

Culture of mouse aortic SMCs (MASMCs)

Primary MASMCs were isolated from mouse aortas from littermates at 2–3 months of age as described previously (Sakata et al. 2004). Cells were plated in complete culture medium DMEM supplemented with 20% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL), and incubated at 37°C in a humidified atmosphere of 5% CO₂. The purity of the cells was verified by immunostaining for both smooth muscle α -actin and calponin (Santa Cruz). MASMCs were used before passage 10 for each experiment.

Western blotting

Cells were lysed with RIPA buffer (50mM Tris pH 7.4, 150 mM NaCl, 0.1 % SDS, 0.5 % sodium Deoxycholate, 1%NP-40, 1 mM EDTA) containing protease inhibitors. The lysates were then separated on sodium dodecyl sulfate–polyacrylamide gels by electrophoresis and transferred onto a Hybond-P membrane. The membranes were blocked with 5% milk in phosphate buffered saline (PBS) with 0.1% Tween 20 for 1 hour at room temperature, and probed with MMP-10 antibody (Millipore) and GAPDH. The signal was visualized with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Total RNA Preparation and Quantitative -Polymerase Chain Reaction (RT-PCR)

Quantitative RT-PCR was performed using an ABI Prism 7500 Fast Real-time PCR System (Applied Biosystems). Total RNA was extracted from cultured smooth muscle cells and A7r5 cells using Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen). Reverse transcription was performed with 2 μ g of RNA using with SuperScript® III First-Strand Synthesis System (Invitrogen). Primers spanning exon-intron junctions were designed to avoid amplification of genomic DNA. PCR conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence changes were monitored with SYBR Green PCR Supermix (Applied Biosystems) after every cycle, and dissociation curve analysis was performed at the end of 40 cycles to verify PCR product (0.5 °C/s increase from 55–99 °C with continuous fluorescence reading). The GAPDH gene was used to normalize samples for comparison. To quantify changes in gene expression, the $\Delta\Delta$ Ct method was used to calculate the relative fold changes as previously described [14]. Primer sets for qPCR were used as following: for GAPDH: 5'-cctcattgacctcaactac-3' and 5'-ggaaggccatgccagtgc-3'; for CHF1: 5'-tgaggagcgagaacaattac-3' and 5'-tttcaaaagctgttgccact-3'.

shRNA Knockdown of CHF1/Hey2

Clones of CHF1/Hey2 shRNA contained within pLKO.1 plasmid containing puromycin resistance were purchased from Open Biosystems (Huntsville, AL). HEK293T cells were transfected with the CHF1/Hey2 shRNA plasmid, a packaging plasmid psPAX2, and an

envelope plasmid pMD2.G using the calcium phosphate precipitate method. Lentivirus particles were collected by centrifugation and titered using an HIV-1 p24 Antigen assay (Advanced BioScience Laboratories, Kensington, MD). A7R5 cells were transduced with CHF1/Hey2 shRNA lentiviral particles at a MOI of 10, followed by selection with 2 μ g/ml puromycin.

MMP-10 activity Assay

MMP-10 activity was measured with a SensoLyte® 520 MMP-10 Assay Kit according to the manufacturer's protocol (ANASPEC). Cultured smooth muscle cells were lysed in assay buffer containing 0.1% (v/v) TritonX-100. MMP-10 was activated by incubating the MMP-10 containing-samples with APMA (4-aminophenylmercuric acetate) for 24 hours at 37°C. Activated enzyme was then mixed with the MMP-10 substrate FAM/QXL™520 FRET peptide. Fluorescence signal was measured at Excitation/Emission=490 nm+20 / 520+20 nm.

Bioinformatic analysis

Approximately 2.5bp of the region upstream of the transcription start site of the mouse *MMP10* gene was screened for putative transcription factor binding sites using TFSEARCH (version 1.3, 1995, Kyoto University, Japan) and MotifMogul (version 1.0, November 2008, Institute of Systems Biology). The taxonomy matrix was entered as 'vertebrate' and the threshold score was set at 90.0 for the analysis.

Statistical analysis

Relative promoter activities were compared by a two-tailed Student's T-test, and a *p*-value<0.05 was considered statistically significant in all tests.

Results

Decreased expression of CHF1/Hey2 results in elevated *MMP10* expression and activity

Our previous study demonstrated that the mRNA expression level of *MMP10* was increased in vascular smooth muscle cells (VSMCs) isolated from CHF1/Hey2 global knockout mice, after treatment with PDGF, as measured by oligonucleotide microarrays [8]. To determine whether changes in CHF1/Hey2 expression lead to changes in *MMP10* expression in the absence of PDGF stimulation, quantitative RT-PCR was performed on RNA isolated from WT and KO SMCs, as shown in Fig1a. Western blot analysis was also used to compare the expression level of the MMP-10 protein between WT and knockout SMCs, as shown in Supplemental Fig. 1a. The results demonstrate that the expression level of *MMP10* mRNA and protein in knockout SMCs is higher than that in WT control cells, implying that CHF1/Hey2 represses *MMP10* expression.

To validate whether changes in *MMP10* expression are direct effects of CHF1/Hey2 in vascular smooth muscle cells, we further compared *MMP10* expression level in untreated and CHF1/Hey2 shRNA treated A7r5 vascular smooth muscle cells. *MMP10* mRNA expression was found to be highly increased when CHF1/Hey2 was knocked down by CHF1/Hey2 shRNA (Fig. 1b). This result is consistent with the observation we had in CHF1 knockout SMCs and implies that increased *MMP10* expression is likely a direct consequence of CHF1/Hey2 loss of function.

Elevated *MMP10* expression in CHF1/Hey2 KO SMCs results in elevated MMP-10 Activity

To determine the functional significance of elevated *MMP10* mRNA and protein in KO SMCs, we tested MMP-10 activity in CHF1/Hey2 knockout mouse aortic SMCs. MMP-10

is normally expressed as an inactive zymogen. The results demonstrated that MMP-10 enzymatic activity was significantly upregulated in CHF1/Hey2 knockout SMCs (Supplemental Fig. 1b).

Identification of constitutive and CHF1/Hey2 responsive elements within the *MMP10* promoter

To study *MMP10* promoter activity, promoter DNA fragments of different lengths (2.5kb, 2kb, 1.5kb, 1kb and 0.5 kb) located immediately upstream of the transcription start site of the mouse *MMP10* gene were cloned from mouse genomic DNA by PCR into the pGL3-Basic luciferase reporter vector. The sequence of each construct was then confirmed by DNA sequencing. These five constructs were transfected into 293T and A7r5 cells, and luciferase activity was then determined. A comparison of luciferase activity revealed that the luciferase reporter plasmid containing the longest promoter sequence (2.5kb) demonstrated the highest luciferase activity (Fig. 2a). Similar results were obtained in A7r5 smooth muscle cells (Fig. 4a). These results suggested that the *MMP10* promoter segment between -2.5kb and -2kb contains elements that confer constitutive activation in both types of cells. Reconstitution of a minimal promoter containing the constitutive 500bp element linked to the 500bp proximal promoter did not restore promoter activity (Fig. 2b), however, indicating that this constitutive element is necessary but not sufficient for full promoter activity.

To examine the possible direct influence of CHF1/Hey2 on *MMP10* promoter activity, we cotransfected a CHF1/Hey2 expression plasmid. As expected, the *MMP10* promoter activity was repressed in 293T cells (Fig. 2a). Surprisingly, CHF1/Hey2 activated the *MMP10* promoter in A7r5 cells (Fig. 4). These observations indicated that CHF1/Hey2 is involved in the transcriptional regulation of *MMP10*, but its precise role in vivo may be complex.

To identify precise nucleotide sequences in the *MMP10* Promoter that confer responsiveness to CHF1/Hey2, we performed a search for regulatory elements for transcription factors in the promoter region of the *MMP10* gene. CHF1/Hey2 has previously been reported to bind the E-box sequence (CANNTG) [15]. Within the 2.5kb proximal promoter, we identified 12 canonical E-boxes (Fig. 2c). To identify their contribution to *MMP10* promoter activity, a mutated *MMP10* reporter plasmid containing mutations in all twelve E-boxes was transfected into both 293T cells and A7r5 cells. Surprisingly, *MMP10* reporter luciferase activity was totally abolished when all the E-boxes are mutated (Fig. 3a).

To better define the importance of these E-boxes for *MMP10* expression, the twelve E-boxes in the 2.5kb luciferase reporter plasmid were mutated individually. Our results showed that all the single E-box mutations in *MMP10* promoter attenuated reporter luciferase activity in 293T cells (Fig. 3b). Mutation of the Site 2 E-box resulted in a significant drop in reporter activity to the basal level of luciferase activity generated by a promoterless pGL3-Basic construct (Fig. 3b). Moreover, mutations in sites 4, 8 and 10 E-boxes individually abolished repression of the *MMP10* promoter by CHF1/Hey2. Furthermore, mutations in sites 5, 7, 9, 11 and 12 led to CHF1/Hey2-dependent activation of luciferase reporter activity (Fig. 3b). In A7r5 cells, however, the individual site 2 and site 12 E-box mutations did not change the reporter luciferase activity significantly (Fig. 4b).

Based on the critical role of the 500bps between -2.5kb and -2kb for *MMP10* constitutive promoter activity, the site 12 E-box located within this region was mutated in combination with each of the other 11 E-boxes. Double mutants combining site 12 and site 2 or site 12 and site 9 conferred the lowest luciferase activity comparable to the promoterless control plasmid (Fig. 3c). Double mutants of sites 12/1, 12/10 and 12/11 restored constitutive activity and abolished repression or activation by CHF1/Hey2. Double mutants in sites 12/3

and 12/5 were similar to mutation in sites 3 and 5 alone. Combined mutation in sites 12/4, 12/6 or 12/8 demonstrated activation by CHF1/Hey2, similar to mutation in site 12 alone. Consistently, the site 12/site 9 combined mutations also decreased reporter luciferase activity in A7r5 cells (Fig. 4c). In A7r5 cells, the combined site 12/ site 5 mutation had no effect on constitutive activity but completely abolished CHF1/Hey2 responsiveness. These results imply that specific E-boxes play a critical role for both constitutive and CHF1/Hey2-dependent *MMP10* promoter activity.

Discussion

In this study, we extended our previously published finding that *MMP10* expression is increased in CHF1/Hey2 knockout SMCs after PDGF treatment by demonstrating that this expression is also increased at the protein level under basal, untreated conditions. In addition, we found that shRNA knockdown of CHF1/Hey2 expression in a smooth muscle cell line A7r5 led to an increase in *MMP10* expression. Furthermore, we demonstrated that MMP-10 enzymatic activity in CHF1 knockout aortic SMCs was increased which is consistent with the increased *MMP10* expression level. To gain further insight into how CHF1/Hey2 regulates the *MMP10* promoter, we have characterized the mouse *MMP10* promoter in reporter assays. A constitutively active promoter element was mapped between -2.5kb and -2kb, and multiple E-boxes were found to be important for the regulation of expression by CHF1/Hey2. Previous studies have shown that a MEF2 site located within the -886 bp promoter is important for constitutive activity in endothelial cells [11] and that two AP-1 sites and two PEA3 sites are responsible for constitutive expression and induction by inflammation [16]. These sites do not appear to be active in smooth muscle cells. Interestingly, a MEF2 binding site is located within the constitutively active promoter element at -2096 to -2075 (Fig. 3c) and may be contributing to activity, but such a contribution would be surprising in the absence of activity of other MEF2 sites known to be active in other cell types.

To identify potential transactivators binding to the promoter region between -2.5kb and -2kb that may confer constitutive activity, we performed additional sequence analysis. The sequence analysis of this region revealed the presence of consensus binding sequences for several transcription factors including STAT1 and AML-1a. STAT1 is a well-known transactivator and is possibly the main activator for this promoter. AML-1a lacks the transactivation domain compared to another two alternative splice variants of the *AML1* gene, AML-1b and AML-1c, and inhibits transcriptional activity [17]. The DNA binding domain for AML-1a overlaps with the site 12 E-box and the mutated site 12 E-box is expected to prevent the binding of AML-1a to the promoter. Our data showing that mutation of the site 12 E-box in -2.5kb to -2kb region enhanced the promoter activity (Fig. 4a) is consistent with a model of co-repression of *MMP10* by CHF1/Hey2 and AML-1a, although further investigation is needed to explore this possibility.

A surprising finding is that CHF1/Hey2 functions as a transcriptional activator in reporter gene assays done in A7r5 cells (Fig. 4) instead of a repressor as is seen in 293T cells. ShRNA directed against CHF1/Hey2 in A7r5 cells leads to increased RNA expression, suggesting that endogenous CHF1/Hey2 functions as a transcriptional repressor. The possible reason for this discrepancy is that other cofactors such as HDACs, histones and other chromatin proteins influence endogenous transcription in ways that are not assessed in our reporter gene assays.

Another interesting result of this study is that single E-box mutations and combinations of E-box mutations in the *MMP10* promoter resulted in CHF1/Hey2-dependent activation of the reporter gene. CHF1/Hey2 has previously been shown to be a transcriptional repressor

[4,5,13,15,18,19,20,21]. Our data indicate the potential novel role of CHF1/Hey2 as a transcriptional activator, which has not been previously reported. A possible explanation may be that CHF1/Hey2 contains both activation and repression domains for transcriptional regulation, although no activation domains have been reported previously. Other possibilities include effects on recruitment of other coactivators in certain contexts, such as the activator STAT1 near the site 12 E-box or through inhibition of the binding of a repressor such as AML1a.

In conclusion, we have identified an important element in the mouse *MMP10* promoter that drives expression in smooth muscle cells that has not been previously reported. In addition, we have determined that E-box sequences in the mouse *MMP10* promoter play an important role in the regulation of *MMP10* expression by CHF1/Hey2. These findings may have important implications for understanding the role of CHF1/Hey2 and *MMP10* in vascular smooth muscle biology and may provide useful information for treatment and prevention of the vascular diseases.

Highlights

- Knockout or knockdown of CHF1/Hey2 in vascular smooth muscle cells leads to increased expression and activity of *MMP10*
- CHF1/Hey2 directly represses the *MMP10* promoter in reporter assays
- Regulation of *MMP10* by CHF1/Hey2 is mediated by multiple E-box elements

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant HL076232 (M.T.C.) and an American Heart Association postdoctoral fellowship (L.W.). M.E.H and F.M. were supported by NIH Training Grant T32 HL07828.

References

1. Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. *Nature*. 2001; 414:216–220. [PubMed: 11700560]
2. Zhong TP, Rosenberg M, Mohideen M-APK, Weinstein B, Fishman MC. *gridlock*, an HLH gene required for assembly of the aorta in zebrafish. *Science*. 2000; 287:1820–1824. [PubMed: 10710309]
3. Watanabe T, Koibuchi N, Chin MT. Transcription factor CHF1/Hey2 regulates coronary vascular maturation. *Mech Dev*. 2010; 127:418–427. [PubMed: 20619341]
4. Doi H, Iso T, Yamazaki M, Akiyama H, Kanai H, Sato H, Kawai-Kowase K, Tanaka T, Maeno T, Okamoto E, Arai M, Kedes L, Kurabayashi M. HERP1 inhibits myocardin-induced vascular smooth muscle cell differentiation by interfering with SRF binding to CArG box. *Arterioscler Thromb Vasc Biol*. 2005; 25:2328–2334. [PubMed: 16151017]
5. Proweller A, Pear WS, Parmacek MS. Notch signaling represses myocardin-induced smooth muscle cell differentiation. *J Biol Chem*. 2005; 280:8994–9004. [PubMed: 15634680]
6. Tang Y, Urs S, Liaw L. Hairly-related transcription factors inhibit Notch-induced smooth muscle alpha-actin expression by interfering with Notch intracellular domain/CBF-1 complex interaction with the CBF-1-binding site. *Circ Res*. 2008; 102:661–668. [PubMed: 18239137]
7. Sakata Y, Xiang F, Chen Z, Kiriya Y, Kamei CN, Simon DI, Chin MT. Transcription Factor CHF1/Hey2 Regulates Neointimal Formation In Vivo and Vascular Smooth Muscle Proliferation and Migration In Vitro. *Arterioscler Thromb Vasc Biol*. 2004; 24:2069–2074. [PubMed: 15345511]

8. Shirvani SM, Mookanamparambil L, Ramoni MF, Chin MT. Transcription Factor CHF1/Hey2 Regulates the Global Transcriptional Response to Platelet-Derived Growth Factor in Vascular Smooth Muscle Cells. *Physiol Genomics*. 2007; 30:61–68. [PubMed: 17327490]
9. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochemical pharmacology*. 2009; 78:539–552. [PubMed: 19413999]
10. Pardo A, Selman M, Kaminski N. Approaching the degradome in idiopathic pulmonary fibrosis. *The international journal of biochemistry & cell biology*. 2008; 40:1141–1155.
11. Chang S, Young BD, Li S, Qi X, Richardson JA, Olson EN. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. *Cell*. 2006; 126:321–334. [PubMed: 16873063]
12. Gill JH, Kirwan IG, Seargent JM, Martin SW, Tijani S, Anikin VA, Mearns AJ, Bibby MC, Anthoney A, Loadman PM. MMP-10 is overexpressed, proteolytically active, and a potential target for therapeutic intervention in human lung carcinomas. *Neoplasia*. 2004; 6:777–785. [PubMed: 15720804]
13. Chin MT, Maemura K, Fukumoto S, Jain MK, Layne MD, Watanabe M, Hsieh C-M, Lee M-E. Cardiovascular basic helix loop helix factor 1, a novel transcriptional repressor expressed preferentially in the developing and adult cardiovascular system. *Journal of Biological Chemistry*. 2000; 275:6381–6387. [PubMed: 10692439]
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
15. Iso T, Sartorelli V, Poizat C, Iezzi S, Wu HY, Chung G, Kedes L, Hamamori Y. HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Molecular & Cellular Biology*. 2001; 21:6080–6089. [PubMed: 11486045]
16. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Molecular and cellular biochemistry*. 2003; 253:269–285. [PubMed: 14619979]
17. Liu X, Zhang Q, Zhang DE, Zhou C, Xing H, Tian Z, Rao Q, Wang M, Wang J. Overexpression of an isoform of AML1 in acute leukemia and its potential role in leukemogenesis, *Leukemia* : official journal of the Leukemia Society of America. *Leukemia Research Fund, U.K.* 2009; 23:739–745.
18. Nakagawa O, McFadden DG, Nakagawa M, Yanagisawa H, Hu TH, Srivastava D, Olson EN. Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:13655–13660. [PubMed: 11095750]
19. Fischer A, Klattig J, Kneitz B, Diez H, Maier M, Holtmann B, Englert C, Gessler M. Hey basic helix-loop-helix transcription factors are repressors of GATA4 and GATA6 and restrict expression of the GATA target gene ANF in fetal hearts. *Mol Cell Biol*. 2005; 25:8960–8970. [PubMed: 16199874]
20. Xiang F, Sakata Y, Cui L, Youngblood JM, Nakagami H, Liao JK, Liao R, Chin MT. Transcription factor CHF1/Hey2 suppresses cardiac hypertrophy through an inhibitory interaction with GATA4. *Am J Physiol Heart Circ Physiol*. 2006; 290:H1997–H2006. [PubMed: 16603706]
21. Shirvani S, Xiang F, Koibuchi N, Chin MT. CHF1/Hey2 Suppresses SM-MHC Promoter Activity Through an Interaction with GATA-6. *Biochem Biophys Res Commun*. 2006; 339:151–156. [PubMed: 16293227]

Fig. 1. Decreased expression of CHF1 promotes increased expression of *MMP10* in SMCs and A7r5 cells

(a) Quantitative RT-PCR analysis of mRNA expression of *CHF1/Hey2* (left panel), and *MMP10* RNA (right panel) isolated from WT and KO SMCs (b) Quantitative RT-PCR analysis of mRNA expression of *CHF1/Hey2* and *MMP10* RNA extracted from CHF1/Hey2 shRNA treated A7r5 cells and untreated cells. Data were normalized to GAPDH RNA expression in the same sample and reported as relative expression (mean $\pm$ SE) compared to levels in wild type control cells (* $p = 0.017$). The experiments were repeated three times and similar results were obtained.

Fig. 2. Mutational analysis of the mouse *MMP10* gene promoter region

(a–b) The structures of a series of *MMP10*-promoter luciferase reporter plasmids are shown schematically (left) and their relative luciferase activities are shown to the right of each construct. Numbers indicate the location of E-boxes relative to the transcription start site. *MMP10* reporter constructs and pCDNA3-CHF1 were transfected into 293T cells. Results are representative of experiments done in triplicate. (c) Sequence analysis of the 2.5 kb upstream promoter sequence of the mouse *MMP10* gene for potential regulatory elements with TFSEARCH (version 1.3, 1995, Kyoto University, Japan) and MotifMogul (version 1.0, November 2008, Institute of Systems Biology). E-boxes are marked in boxes. Putative recognition sites for other transcription factors are underlined and labeled.

Fig. 3. E-boxes mediate both constitutive and CHF1/Hey2-dependent MMP10 promoter activity in 293 T cells

(a) Single E-box mutations were introduced into the 2.5kb promoter fragment and assessed for luciferase activity in 293T cells, in the presence of either the expression vector pcDNA3 or pcDNA-hCHF1. (b) E-box mutations in the site 12 E-box were combined with mutations in each of the other E-boxes and assessed for luciferase activity in 293T cells, in either the absence or presence of CHF1/Hey2. The activity was normalized to β -galactosidase activity induced by cotransfection with the pcDNA/Hygro/LacZ plasmid. The assays were carried out in triplicate to confirm the reproducibility of the observations and the error bars indicate the standard error of the mean. *indicates significant changes in the presence of exogenous

CHF1/Hey2, $p<0.05$. # indicates significant changes in the luciferase activities of all E-box mutated promoters in comparison to the wild type promoter, $p<0.05$.

Fig. 4. Analysis of *MMP10* promoter regulation in A7r5 cells

(a) Deletion mutants of the *MMP10* promoter were transfected into A7r5 smooth muscle cells. (b) Selected single E-box mutants were transfected into A7r5 cells in the absence or presence of CHF1/Hey2. (c) E-box mutations in the site 12 E-box were combined with selected mutations in other E-boxes and assessed for luciferase activity in A7r5 cells, in either the absence or presence of CHF1/Hey2. The assays were carried out in triplicate to confirm the reproducibility of the observations and the error bars indicate the standard error of the mean. *indicates significant changes in the presence of CHF1/Hey2, $p < 0.05$.