Transcription factor CHF1/Hey2 regulates the global transcriptional response to platelet-derived growth factor in vascular smooth muscle cells

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Submitted 14 December 2006; accepted in final form 23 February 2007

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 30: 61–68, 2007. First published February 27, 2007; doi:10.1152/physiolgenomics.00277.2006.—The cardiovascular restricted transcription factor CHF1/Hey2 has been previously shown to regulate the smooth muscle response to growth factors. To determine how CHF1/Hey2 affects the smooth muscle response to growth factors, we performed a genomic screen for transcripts that are differentially expressed in wild-type and knockout smooth muscle cells after stimulation with platelet-derived growth factor. We screened 45,101 probes representing >39,000 transcripts derived from at least 34,000 genes, at eight different time points. We analyzed the expression data utilizing an algorithm based on Bayesian statistics to derive the best polynomial clustering model to fit the expression data. We found that in a total of 9,827 transcripts the normalized ratio of knockout to wild-type expression diverged more than threefold from baseline in at least one time point, and these transcripts separated into 17 distinct clusters. Further analysis of each cluster revealed distinct alterations in gene expression patterns for immediate early genes, transcription factors, matrix metalloproteinases, signaling molecules, and other molecules important in vascular biology. Our findings demonstrate that CHF1/Hey2 profoundly affects vascular smooth muscle phenotype by dramatically altering the absolute expression level of a variety of genes and the kinetics of growth factor-induced gene expression.

Microarray; genomics; vascular biology; gene knockout

ARTERIOSCLEROSIS IS THE LEADING cause of death in developed countries. The pathogenesis of arteriosclerosis is complex, involving the interaction of multiple cell types, genetic factors, soluble and matrix components, and derangements in blood flow (for review, see Ref. 17). The vascular smooth muscle cell (VSMC) is a major component of the arterial wall and is thought to play a critical role in the development of arteriosclerosis (7, 20, 27). In normal vessels, VSMCs are quiescent, differentiated, and contractile and function to maintain vascular tone and blood pressure. In response to injury, whether mechanical, chemical, or immunologic, they develop a dedifferentiated, proliferative, and secretory phenotype as they migrate from the media to the neointima (7, 20, 27). The upstream regulators that affect expression of these various genes in this context are largely unknown.

We have previously cloned the basic helix-loop-helix (bHLH) protein CHF1/Hey2 (2). Studies in the adult vasculature have suggested that CHF1/Hey2 gene expression varies after vascular injury and may be important for smooth muscle cell (SMC) growth and protection against apoptosis (34, 35). Mice with deletion of CHF1/Hey2 have thin-walled arteries (29) and manifest a functional vascular phenotype in which neointimal formation after vascular injury is decreased and smooth muscle cells (SMCs) from these mice are defective in their responses to the growth factors platelet-derived growth factor (PDGF) and heparin-binding epidermal growth factor-like growth factor (30). We and others have also shown that CHF1/Hey2 can inhibit expression of smooth muscle-specific genes (5, 23, 31). A comprehensive analysis of the CHF1/Hey2-dependent genome in the adult vasculature as a method to examine its potential role in the pathogenesis of occlusive vascular diseases has not yet been performed.

To determine the potential transcriptional mechanisms by which CHF1/Hey2 regulates the growth factor response in SMCs, we performed a genome-wide dynamic microarray screen to characterize and compare changes in the transcriptional programs of wild-type and CHF1/Hey2 knockout (−/−) VSMCs following treatment with PDGF, as a function of time. Following cluster analysis using Bayesian dynamics, we have identified numerous potential effector genes whose dynamic gene transcription patterns are profoundly altered after PDGF treatment.

Previous transcriptional profiling studies of VSMCs have been performed to identify transcripts that distinguish arterial from venous SMCs (15), that distinguish plaque VSMCs from medial SMCs (19), that establish SMC identity in an in vitro model of smooth muscle differentiation (32), and that are differentially expressed in rats susceptible to hypertension (6). In each case, static comparisons were made. Our study is the first to systematically perform a comparative time-series analysis of gene expression in CHF1/Hey2 knockout cells to identify transcriptional alterations that affect vascular smooth response to growth factors. Here we report that knockout of a single gene, CHF1/Hey2, alters the growth factor-induced phenotype of SMCs by dramatically altering the dynamic expression of a large number of genes.

MATERIALS AND METHODS

Culture of mouse aortic SMCs, PDGF treatment, and RNA harvest. Preparation of aortic SMCs for culture has been described (30). The purity of the cells was verified by immunostaining for both smooth muscle α-actin and calponin. Fibroblast contamination was not de-
detectable. Wild-type and knockout cells were prepared at the same time from littermates at 8–10 wk of age. Two independent preparations of each genotype were tested for their differential proliferative and migratory response to PDGF, as previously described (30). Each knockout cell preparation demonstrated defects in the proliferative and migratory response to PDGF, as expected. For these experiments, the mouse aortic smooth muscle cells (MASMCs) were between passages 10 and 20, but RNAs were prepared from cells at the same passage. For PDGF induction, cells were grown to 95% confluence on 150-mm plates, washed four times with phosphate-buffered saline, and serum starved with DMEM supplemented with 0.4% FCS. After 48 h in a 37°/5% CO2 humidified incubator, the cells were washed four times with phosphate-buffered saline and then treated with either DMEM or DMEM supplemented with 5 ng/ml human PDGF-BB. Cells were then incubated in the humidified incubator for the length of time indicated by the experiment. After the appropriate length of treatment, the cells were washed one time with phosphate-buffered saline, and RNA was harvested with the Qiagen RNeasy protocol according to the manufacturer’s instructions (Qiagen).

For the microarray experiment, RNA was harvested from knockout and wild-type MASMCs at 0 (no PDGF-BB treatment), 15, 30, 60, 120, 240, and 480 min following stimulation with PDGF-BB. This mRNA was reverse transcribed into cDNA that was then used as a template for in vitro transcription and biotin labeling of a cRNA target population. The resulting mixtures from each time point were hybridized to Affymetrix Mouse Expression Set 430 microarrays consisting of 45,101 probes corresponding to >39,000 transcripts, followed by staining with a streptavidin-phycoerythrin conjugate. The chips were then washed in a fluids station and scanned with a GeneArray Scanner (Hewlett-Packard) to collect fluorescence intensity data. All microarray raw data were deposited in the Gene Expression Omnibus database under accession number GSE6526.

Microarray data analysis. Knockout-to-wild-type ratios of gene expression were calculated for each probe at each time point and then normalized to the expression ratio at the zero time point to generate expression profiles. Cluster analysis of the expression profiles was performed with Bayesian analysis using CAGED software (26). The Bayesian approach assumes that the observed data were generated by a set of unknown processes without assumptions regarding the number of processes. All clustering models were initially assumed to be equally probable (i.e., a uniform prior distribution over these models was assumed). The software then calculated the posterior probability of each model given the observed data and selected the most probable.

Genes grouped this way into a single cluster were considered to be members of each cluster. The natural logarithm of the normalized ratio of knockout to wild-type gene expression as a function of time for each cluster is shown in Fig. 1. A complete listing of all differentially expressed genes and the statistical goodness of fit for each cluster is available online (http://www.hpcgg.org/biofx/Chin/median/3fold/poly63foldmedian.html). Gene Ontology annotations for each member of each cluster were downloaded from a publicly available online data resource (http://db.chip.org/unchip/unchip-top). Members of each cluster were sorted by Gene Ontology classification and then reviewed manually. Those that had particular relevance to vascular biology, cell signaling, or transcription were examined in more detail.

Each cluster demonstrated distinct characteristics. Cluster 1, for example, was notable for an dynamic changes in the knockout to wild-type expression ratio over time, where the ratio of knockout to wild-type gene expression initially increased at early time points but ultimately decreased to below baseline at the end of the time course. Many members of this cluster fall into the category of “immediate early genes” that were originally described as genes that are rapidly induced after serum stimulation and do not require protein synthesis for induction (14). Examples include c-fos, c-jun, egr2, ier2, KLF10, ier3, ier5, and egr3. Other members relevant to vascular biology include thrombospondin-1, factor III (tissue factor), osteomodulin, and Itag5 (integrin αV). Transcription factors include KLF2, KLF9, NFIL3E4BP4-like, Bhlhb2, INAP, Copeb, Tieg, egr2/Krox20, c-jun, Nr4a1, c-fos, ATF3, and junB. A representative transcriptional ratio over time is shown for c-jun, which shows a much greater induction in the knockout cells initially, but a delayed increase in the wild-type cells at the end of the time course (Fig. 2A). These findings indicate that both the magnitude and the timing of the cellular response to PDGF is altered. While the majority of the cluster shows a similar profile, the degree of induction and relative expression at each time point varies considerably, which probably reflects the sensitivity of the clustering method. Another representative example is egr2, which also shows earlier induction in the knockout cells and delayed induction in the wild-type cells (Fig. 2B). Alteration in the response of immediate early genes suggests that absence of CHF1/Hey2 alters the ability of cells to respond to a variety of growth factor stimuli.

Cluster 2 is also notable for alteration in the expression dynamics of gene expression. Knockout gene expression again shows an early relative increase, a later relative decrease, and then converges with wild-type gene expression at later time points. The members of this cluster include a variety of structural genes, such as smoothelin, tropomodulin, tubulin 4, syndecan 4, and vasodilator-stimulated phosphoprotein (VASP) (1). VASP is of particular interest because it has previously been shown to play a role in vascular biology, as an integrator of the signaling response. Its expression pattern is shown (Fig. 2C). Other potentially important members of this cluster include the matrix metalloproteinase inhibitor tissue inhibitor of metalloproteinase (TIMP) 3, the integrin signaling molecule Bcar1, and the transcriptional regulators serum response factor (SRF), Foxe2, and CARP. SRF has previously been shown to play important roles in both the growth factor response and muscle specific gene expression (36). Analysis of the SRF expression pattern is shown in Fig. 2D and demonstrates that the overall induction of SRF is blunted in the knockout compared with wild-type cells.
Cluster 3 is most notable for differential expression of the transcriptional regulators ATF5β, MafB, HSAz, Zik1, zfp161, nucleolin, zfp97, and zfp235. Cluster 4 also is notable for differential regulation of primarily transcriptional regulators, including Hoxb8, Hes5, Tead3, Ipa, Bcl11a, and Rel. Cluster 5 is most striking for including the architectural transcription factors hmga1 and hmga2 and the extracellular matrix regulators TIMP, matrix metalloproteinase (mmp) 10, and mmp13. These architectural transcription factors have been implicated in cell growth and the development of benign mesenchymal tumors.

![Fig. 1. Clusters of dynamic gene expression profiles of vascular smooth muscle cell after platelet-derived growth factor (PDGF) stimulation. The data are plotted as the natural logarithm of the normalized ratio of knockout (KO) cell gene expression to wild-type (WT) cell gene expression for each probe vs. time in minutes. Expression profiles for 17 clusters representing 9,827 genes are shown.](image)

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![Fig. 2. Expression profiles for representative genes from clusters 1 and 2. Data are presented showing the raw expression profile for KO and WT cells over time at top and the natural logarithm of the normalized individual expression ratios over time at bottom for cluster 1 members c-jun (A) and egr2 (B). A similar analysis is shown for the cluster 2 members vasodilator-stimulated phosphoprotein (VASP, C) and serum response factor (SRF, D). Solid line in the bottom panels, normalized expression ratio.](image)
and are induced in vascular smooth muscle cells after wire injury in vivo and serum stimulation in vitro (3). Interestingly, they have also been described as “delayed early genes” induced several hours after growth factor stimulation, requiring protein synthesis (13). Analysis of their transcriptional expression kinetics demonstrates that their induction is delayed in the knockout cells (Fig. 3, A and B), raising the possibility that the delayed early response to growth factors is altered in these cells. Induction of mmp10 and mmp13 is also delayed in the knockout cells (Fig. 4, A and B), which would suggest a lack of invasiveness and may partially explain the lack of migration through a collagen-coated matrix and diminished neointimal formation observed in vitro and in vivo (30).

Cluster 6 contains a significant contingent of transcription factors, such as Zfp288, Sp1, Foxg1, Tip5, mtf2, pbx2, mafg, copeb, sox4, ssbp2, CBP, zfp30, zfp161, Gli5. It also contains a number of signaling molecules, including Ramp2, Dkk1, snb2, Gna13, il6ra, Has1, Pip5k1b, Ierepo4, Gpr83, fsc1, prk A anchor protein 4, PQL4LARE, EMK, v1rb4, and ArhGAP5. The dynamic expression profile for cluster 6 is also interesting, because it is primarily positive, reflecting a general increase in expression in the knockout cells compared with wild-type. Since CHF1/Hey2 is known to be a transcriptional repressor, members of this cluster may be direct targets of CHF1/Hey2. The dynamic expression profiles of Has1, a biosynthetic enzyme for the extracellular matrix component hyaluronan, and Ramp2, a modulator of calcitonin signaling, are shown (Fig. 4, C and D).

Cluster 7 includes the transcription factors Runx1, Tfdp1, Elk3, Tcf4, jerky, gecd, and zfp207, the cell cycle or cell growth-related genes cyclin H, Vegf-c, Rgs2, c-mer, Fyn, cdc25a, and the extracellular matrix regulators Timp3 and mmp3. Cluster 8 also includes numerous transcription factors such as Id2, CRTR-1, SMAD6, Id3, Id4, hmgb2, hmg2, tle2, rb2, hmgb3, Hoxa7, Hoxb6, Klf9, prrx1, Dbp, Tead2, Klf7, Irx3, Klf4, zfp95, Crem, GATA6, phx1b, Mef2c, per1, per2, nr4a2, TCF4, RPL13a, and MITF-2A. Timp3 and mmp3 are directly relevant to vascular biology for their roles in protection against vascular inflammation (9) and plaque stabilization (12). Their dynamic expression patterns are shown (Fig. 5, A and B). GATA6 is also relevant, as it has been postulated to maintain the differentiated, contractile phenotype of vascular smooth muscle and prevent neointimal formation after injury (18, 22). We have previously shown that CHF1/Hey2 can directly interact with GATA6 and inhibit smooth muscle myosin heavy chain gene expression (31). Analysis of the GATA6 dynamic expression pattern shows that there is a delay in GATA6 downregulation in the knockout cells (Fig. 5C), which would be consistent with our previously reported phenotype in which
mice lacking CHF1/Hey2 demonstrate decreased neointima formation after injury (30).

Cluster 9 is by far the largest cluster, containing 5,357 members. The individual members in each category are too numerous to list, but their distribution by functional class is shown (Fig. 6A). One large subgroup consists of transcription factors and molecules involved in replication and translation. Some interesting transcription factor genes are the forkhead proteins Foxa2, Foxo1, and Foxp1, which are known to play some roles in regulating cellular responsiveness to growth factor signaling (24, 25). Other molecules relevant to vascular biology include thrombomodulin (16) and vcam1 (4), and their dynamic expression profiles are shown (Fig. 6, B and C). It is of interest to note that although these genes are in the same cluster by virtue of similar alterations in expression ratios over time, their individual dynamic expression values diverge significantly.

Cluster 10 is notable for the inclusion of multiple molecules that play roles in signal transduction and receptor signaling, such as Hax1, Bcap37, Ssr4, Dnaj3, Eif4ebp1, Itgb4bp, and Dok1. Cluster 11 is notable for numerous transcription factors such as Tafl0, Rbbp7, Ncor2, Gli5, Etv6, Srebfl, Nfe2l2, Sali2, Nfya, Nfkb2, Hdac1, Hdac3, Sap18, zf263, Nrd16, Sra1, hdc7a, Alrp, Relb, and Tcerg1. This cluster also includes a number of proapoptotic molecules such as Bnip3, Bnip2, Pdcd6ip, Rnp7, and Sh3glb1 and several tyrosine kinases, including Yes, Ilk, Map2k1, Chuk, and Prpk. Cluster 12 is notable for the cell cycle molecules cyclin D1, E2f6, mcm7, Rad1, Rgs2, Pin1, calm1, and Ppm1g, the transcription factors Gtf2h2, Gabpa, Hmx3, Mirf3, Preb, Klf5, Rpo2-3, Rpo1-3, Sdcag33, Miz1, Ppar, Dr1, Gtf2h1, and the signaling molecules Mif, Pdgfa, Il1r, Map3k7 (TAK1), Map2k2, Jak2, and Hras1. Clusters 13 and 14 do not appear to be overrepresented by any particular gene ontology classification. Cluster 15 is notable for the differential expression of the transcription factors Fkhr, Prrx2, Atf2, Ahr, Hif1a, Fosl2, Bcl3, Rnp2c, Nrbf2, Taf7, Atf7ip, Pias1, and the tyrosine kinases Limk2, stk25, Ck4, Pak1, Pak3, Bmpr1a, Prkx, Cdk5, Tgfbr1, Mapkapk2. Cluster 16 contains the transcription factors Snai2, Hoxb5, Foxp1, Klf15, and Nr2f2. Cluster 17 is notable for differential expression of the cell adhesion molecules Vcam1, col15a1, Fln14, and Cspg2. The other members of these clusters are functionally diverse.

Confirmation of candidate genes. Several candidate genes were examined with qRT-PCR to confirm microarray results: Itga5, Has1, Mbd1, PGHS-B, Srf, and VASP. RNA was harvested from independently replicated samples at the initial time point and 2 h after treatment with PDGF-BB. Levels of mRNA for the candidate genes and an internal control, α-tubulin, were measured by a qRT-PCR assay with expression normalized to tubulin mRNA. For all of these genes, the trend observed in the microarray data was reiterated in the qRT-PCR experiment (data not shown).

DISCUSSION

In this experiment, we sought to identify genes in VSMCs whose expression in response to growth factor stimulation is altered in the absence of CHF1/Hey2. To accomplish this objective, we compared the transcriptomes of CHF1/Hey2 knockout and wild-type VSMCs at eight time points following PDGF stimulation and mined the resultant data for genes whose expression varied significantly from the baseline ratios between the two groups of cells. Data mining from our experiment yielded a wide variety of candidate genes whose expression was significantly different between CHF1/Hey2 knockout and wild-type cells. The number and diversity of genes that are differentially expressed were initially surprising. These findings, however, reflect both the sensitivity and power of the CAGED algorithm (26) for determining differential expression in time series analysis through Bayesian statistical techniques. They also demonstrate that the absence of CHF1/Hey2 leads to profound alterations in growth factor-induced gene expression and consequently is an important regulator of the cellular response to growth factors. The tremendous divergence in gene expression patterns is likely due in part to the early temporal disruption of gene expression coupled with the large numbers of transcription factors that are affected. Disruption of the immediate early gene response to growth factors would be expected to have profound effects on genetic cascades that are dependent upon these early factors. There are many transcrip-
tion factors that show altered expression, and since each transcription factor is expected to regulate multiple target genes, the extensive changes in gene expression observed over the course of the experiment are not unexpected. Of note also is that many genes did not show significant differences at the beginning or end of the time course but showed alterations in the kinetics and dynamics of gene expression profiles over time. These findings underscore the importance of time-series analysis of gene expression to identify and dissect important transcriptional cascades in regulating cellular responses to various stimuli. These data also provide insight into the transcriptional changes that underlie the smooth muscle response to a potent cellular mitogen and provide a unique and valuable dataset to explore the connection between extracellular binding of PDGF and intracellular gene regulation by CHF1/Hey2.

Many of the identified genes have roles in vascular smooth muscle function, and their expression profiles are consistent with our previously observed effects of CHF1/Hey2 loss of function on vascular smooth muscle phenotype (30). For instance, the hyaluronan synthase 1 (Has1) gene was especially sensitive to the presence of CHF1/Hey2. There was significant upregulation of Has1 in response to PDGF in the knockout cells that was absent in the wild-type cells. This suggests that CHF1/Hey2 functions to keep Has1 expression suppressed when the cell undergoes growth factor stimulation. It has been reported that PDGF induces the production of hyaluronan by VSMCs, which in turn exerts a potent suppressor effect on VSMC proliferation (21). Our data suggest that one mechanism by which absence of CHF1/Hey2 affects smooth muscle proliferation in vivo is by promoting the synthesis of excess free hyaluronan compared with wild-type cells, which in turn leads to decreased relative proliferation. Further study would be required to confirm the hypothesis, but nonetheless it stands as an exciting potential therapeutic pathway for modulating arteriosclerosis. Many other molecules important in the vessel wall (e.g., VASP, mmp10, mmp13, TIMP, thrombomodulin, etc.) have also been identified in our dataset, as described above.

The dataset derived from this experiment facilitates the generation of many testable hypotheses that will have important ramifications for understanding vascular smooth muscle function and CHF1/Hey2-dependent gene regulation. Thus far CHF family members have only been shown to influence gene transcription indirectly by binding and regulating other transcription factors in the nucleus such as GATA1 (8), GATA4

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![Figure 6](image)

Fig. 6. Functional diversity among annotated genes in cluster 9 (A). Cluster 9 is the largest cluster, containing 5,357 members. A significant number are involved in transcription and translation, which may explain the large number of differentially expressed genes overall. Expression profiles for vascular molecules in cluster 9 (B, C). Data are presented as described in the legend to Fig. 2. Thrombomodulin (B) and vcam1 (C) show a general increase in expression in the KO cells. Thrombomodulin is known to promote anticoagulation, while vcam1 is known to promote inflammatory cell adhesion.
we previously performed a static comparison of baseline gene expression in untreated cells, using three independent RNA samples for each cell type, prior to the initiation of the current study. Although we were able to identify numerous transcripts that are differentially expressed, none were directly related to PDGF signaling, other than SOS1 (M. T. Chin, unpublished results). The SOS1 expression ratio, interestingly, does not vary significantly over the time course of the study, and it does not appear in any of the clusters. The current study, in conjunction with our unpublished results, clearly demonstrates that dynamic differences in gene expression may be at least as important as baseline changes in defining the transcriptional response to growth factors.

An essential question raised by our study is one of how the various changes in dynamic gene expression observed aid in our understanding of the vascular phenotype of CHF1/Hey2-deficient mice. We have previously demonstrated that these mice show defects in PDGF-induced proliferation and migration (30). Our current data demonstrate gene expression alterations in a number of transcription factors associated with immediate early responses to growth factors (c-jun, egr2, SRF), delayed early responses to growth factors (HMGA1, HMGA2), and various molecules associated with the development of occlusive vascular lesions (VASP, mmp10, mmp13, TIMP3, mmp3, GATA6, thrombomodulin, and VCAM1). These findings suggest that the knockout phenotype ensues from perturbation of a complex transcriptional network controlling essential aspects of the smooth muscle cellular response to growth factors and vascular remodeling.

Our experiment was conceived to identify the transcriptional pathways altered in SMCs lacking CHF1/Hey2 that affect smooth muscle behavior during growth factor stimulation. Through dynamic microarray profiling, data mining, and qRT-PCR validation, we have successfully identified numerous genes that may be important in these pathways. The top candidates, along with their cluster number and Gene Ontology classification, are listed in Table 1. Further experimentation to explore the many hypotheses generated will be facilitated by this dataset. The number of genes differentially expressed in CHF1/Hey2 knockout cells and their broad functional and structural diversity demonstrate a significant role for CHF1/Hey2 in VSMC function. Additional characterization of these transcriptional pathways will prove useful for understanding vascular smooth muscle function in health and disease.

GRANTS

This work was supported by National Institutes of Health Grants HL-076232 to M. T. Chin and HG-003354 to M. F. Ramoni. S. M. Shirvani was supported by a fellowship from the Sarnoff Foundation.

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CHF1/Hey2 regulates global transcription


