Mediator Subunit MED28 (Magicin) Is a Repressor of Smooth Muscle Cell Differentiation

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Magicin, a protein that we isolated earlier as an interactor of the neurofibromatosis 2 protein merlin, was independently identified as MED28, a subunit of the mammalian Mediator complex. Mediator complex is an evolutionarily conserved transcriptional cofactor, which plays an essential role in positive and negative gene regulation. Distinct Mediator subunit composition is thought to contribute to gene regulation specificity based on the interaction of specific subunits with subsets of transcription factors. Here we report that down-regulation of Med28 expression in NIH3T3 cells results in a significant induction of several genes associated with smooth muscle cell (SMC) differentiation. Conversely, overexpression of MED28 represses expression of SMC genes, in concordance with our knockdown data. More importantly, multipotent mesenchymal-derived murine precursors can transdifferentiate into SMCs when Med28 is down-regulated. Our data also show that Med28 functions as a negative regulator of SMC differentiation in concert with other Mediator subunits including Med6, Med8, and Med18 within the Mediator head module. Our results provide strong evidence that MED28 may function as a scaffolding protein by maintaining the stability of a submodule within the head module and that components of this submodule act together in a gene regulatory program to suppress SMC differentiation. The results presented here demonstrate for the first time that the mammalian Mediator subunit MED28 functions as a repressor of SMC differentiation, which could have implications for disorders associated with abnormalities in SMC growth and differentiation, including atherosclerosis, asthma, hypertension, and smooth muscle tumors.

We previously identified a novel protein, which we named magicin, as a specific binding partner for the cytoskeletal neurofibromatosis 2 tumor suppressor protein merlin. These two proteins interact in vitro and in vivo and colocalize beneath the plasma membrane. Magicin is a ~24-kDa protein expressed in many cell lines and tissues. Similar to merlin, magicin associates with the actin cytoskeleton as determined by cofractionation, immunofluorescence, and electron microscopy. Our results suggest a role for magicin in receptor-mediated signaling at the cell surface and its possible involvement in the regulation of cytoskeletal reorganization (1). Our recent work also documents that magicin associates with Src-family kinases such as Fyn, Src, and Lck and is phosphorylated by Lck in the T lymphocyte leukemia Jurkat cell line upon CD3 stimulation (2).

Magicin was independently identified as a differentially expressed gene in endothelial cells and named endothelial-derived gene (EG-1) (3). EG-1 expression is elevated in several tumors, and overexpression of EG-1 increases cell proliferation (4, 5). Surprisingly, and more importantly, magicin was also identified as a subunit (MED28) within the mammalian Mediator complex, which functions in the nucleus to regulate transcription (6, 7). The Mediator complex, originally identified in yeast, was subsequently isolated in mammalian cells comprising 30 subunits. Mediator is an important component of the RNA polymerase II transcriptional machinery and plays a crucial role in the activation and repression of eukaryotic mRNA synthesis (8–10). Thus magicin, which we will hereafter refer to as MED28, may serve as a multifaceted adaptor/scaffolding protein to relay cellular signals to the cytoskeleton and from the cytoskeleton to the nucleus.

Mediator exists in at least two forms, differing mainly by the presence or absence of a negative regulatory submodule consisting of cyclin-dependent kinase 8 (CDK8),3 cyclin C, MED12, and MED13 (9, 11). Different Mediator subunits are targets for interactions with various DNA-binding transcriptional activators. Mediator complexes with the same subunit composition are proposed to have distinct activities through interactions with different transcriptional activation domains (10, 12). In addition, studies addressing the functions of the intact mammalian Mediator complex, as well as individual subunits, are beginning to emerge (13, 14).

To evaluate whether MED28 plays a role in controlling specific transcriptional programs and to determine putative target genes regulated by MED28, we performed whole genome

3 The abbreviations used are: CDK8, cyclin-dependent kinase 8; SMC, smooth muscle cell; SM-MHC, smooth muscle-myosin heavy chain; SM-α-Actin, smooth muscle-α-Actin; SM-γ-Actin, smooth muscle-γ-Actin; CRP1, cysteine-rich protein 1; RNAi, RNA interference; siRNA, small interfering RNA; GFP, green fluorescent protein; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, human; KD, knockdown.
expression profiling of Med28 RNAi knockdown in NIH3T3 cells and observed that Med28 suppression results in a significant induction of many genes involved in smooth muscle cell (SMC) differentiation. Overexpression of MED28 represses the expression of SMC genes consistent with our RNAi knockdown data. Intriguingly, our results also show that Med28 suppression in the multipotent mesenchymal-derived murine precursor cell line C2C12 results in transdifferentiation into SMCs, a differentiation pathway not previously associated with this cell line. Furthermore, induction of SMC differentiation by Med28 knockdown involves destabilization of several other subunits in the mammalian Mediator head module. The results presented here demonstrate for the first time that mammalian Mediator subunit MED28 functions as a repressor of SMC differentiation, and this observation could have wide medical implications since abnormalities in SMC growth and differentiation are linked to many disorders, including atherosclerosis, asthma, hypertension, and smooth muscle tumors.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Human full-length MED28 (hMED28) was cloned into the pEGFP-N1 (Clontech) plasmid, as well as the CSCW2 lentiviral plasmid. The CSCW2 vector, packaging, and viral supernatants were provided through the Neuroscience NINDS, National Institutes of Health, Vector Core at Massachusetts General Hospital. For RNAi, SMARTpool® reagents were obtained from Dharmacon RNA Technologies and included murine Med28 (NM_025895; catalog number M-063448-00) and Med6 (NM_027213; catalog number M-055846-00), individual siRNA reagent for murine Med28 (accession number D-063448-02), as well as non-targeting siControl (catalog number D-001206-13-05).

Primary antibodies used included the anti-MED28 antibodies Tim3 (rabbit polyclonal) and 7E1 (mouse monoclonal), which have previously been described (1); M2 (anti-FLAG monoclonal; Sigma), MED6, CDK8, and SM22α/Transgelin (Santa Cruz Biotechnology); SM-α-Actin (MP Biomedicals); GAPDH (Chemicon); and SM-α-Actin, α-tubulin, and normal rabbit serum (Sigma). Rabbit polyclonal antibodies for MED8, MED17, and MED18 were a kind gift from Drs. R. C. Conaway and J. W. Conaway, and a second MED17 rabbit polyclonal antibody was a kind gift from Dr. R. G. Roeder. Secondary antibodies included horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Amersham Biosciences) and horseradish peroxidase-conjugated anti-goat (Santa Cruz Biotechnology) for Western analysis and Alex Fluor 594-phalloidin (Molecular Probes) and Cy3-donkey antirabbit for immunofluorescence.

**Cell Culture**—The mouse fibroblast line NIH3T3 was maintained in Iscove’s Dulbecco’s modified Eagle’s medium (Cellgro) containing 10% fetal bovine serum (Sigma). The multipotent, mesenchymal-derived C2C12 line was cultured at ≤60% confluency in growth medium containing Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 20% fetal bovine serum (Sigma) to prevent myoblast differentiation.

**Transfection and Infection**—All transfections were performed using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s protocol, utilizing reverse transfection methods to increase transfection efficiency. For rescue experiments, C2C12 cells were transfected with either small interfering control or siRNA-mMed28 plus GFP-hMED28 plasmid. Transfections were performed at 48 h (for NIH3T3) and 72 h (for C2C12) after transfection. Lentiviral infections were carried out in NIH3T3 cells using low titer viral supernatants of either CSCW2 vector alone or hMED28-CSCW2 at a multiplicity of infection of 100 transforming units/cell. At 72 h after infection, cells were analyzed for enhanced GFP expression and fluorescence-activated cell sorter-sorted by the Analytical and Quantitative Cytology Core Facility at Massachusetts General Hospital (MGH).

**Nuclear Fractionation, Co-immunoprecipitation, and Western Blotting**—Isolation of nuclear fractions was carried out using a modification of the Dignam et al. method (28). Co-immunoprecipitation (co-IP) was carried out as described previously (1). Briefly, for endogenous co-IP of Med28 and Med6, NIH3T3 lysates were incubated with anti-MED28 (Tim3), anti-MED6, or normal rabbit serum (as control), along with protein A (MED28 and normal rabbit serum)- or protein G (MED6)-Sepharose (Roche Diagnostics). For co-IP from NIH3T3 nuclear extracts (containing 0.42 m NaCl), extracts were diluted with an equal volume of Dignam Buffer A and then subjected to co-IP using anti-CDK8, anti-MED7, anti-MED17, anti-MED8, and normal rabbit serum as a negative control.

Western blotting was carried out as described previously (1). Briefly, lysates were quantitated, and equal amounts (50–100 μg) were separated by SDS-PAGE, transferred to nitrocellulose Trans-Blot transfer medium (Bio-Rad), and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Immunoblots were visualized using the ECL system (Amersham Biosciences).

**Expression Profiling**—Total RNA was harvested using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Quality control of the RNA (Agilent bioanalyzer) and hybridization on mouse 430 2.0 chips from Affymetrix were performed by the MGH Cancer Center DNA Microarray Core Facility. The results were analyzed using the GeneSifter software (vizXlabs) and the GeneChip® operating software (GCOS) from Affymetrix.

**Real-time RT-PCR and Standard RT-PCR**—First-strand cDNA was synthesized from total RNA using the SuperScript first-strand synthesis system (Invitrogen). Real-time RT-PCR was carried out to analyze Med28 expression, as well as the SM differentiation cofactor cysteine-rich protein 1 (CRP1) (Csrp1) and SM differentiation markers including SM22α (Tagln), SM-α-Actin (Acta2), and SM-α-Actin (Actg2). To perform real-time RT-PCR, first-strand cDNA was combined with iQ SYBR Green supermix (Bio-Rad) and mouse sequence-specific primers for Med28, CRP1, SM22α, SM-α-Actin, and SM-α-Actin, as well as the control housekeeping gene GAPDH. Reactions were carried out using a Bio-Rad iCycler and analyzed with MIQ detection system and software. Fold changes in gene expression were calculated using the comparative Ct (threshold cycle) method.

Standard RT-PCR using mouse-specific primers was also carried out for 27–35 cycles to visualize changes in expression for Med28, SM22α, SM-α-Actin, SM-α-Actin, CRP1, and GAPDH, as well as the definitive smooth muscle marker SM.
myosin heavy chain (SM-MHC). Primer sequences are provided in supplemental Table 1. Mouse-specific primers for SM-MHC have been previously described (29).

RESULTS

Knockdown of Med28 in NIH3T3 Cells Reveals Up-regulation of a Set of SMC Genes—In an earlier study where we isolated Med28 (magician) as a novel merlin-interacting protein, we observed Med28 in association with the actin cytoskeleton of the mouse neuronal (Cath. a-differentiated) cell line as determined by cofractionation, fluorimicroscopy, and electron microscopy (1). MED28 has also been identified as a subunit of the mammalian Mediator complex functioning in the nucleus as a transcriptional co-regulator (6, 7). Thus MED28 is a unique Mediator subunit with possible functions both in the cytoskeleton and in the nucleus.

In an effort to identify the genes that are regulated by Med28, we compared the gene expression profiles in NIH3T3 cells where Med28 expression was knocked down by RNAi, versus a non-targeting siRNA control, by microarray analysis. siRNA-mediated knockdown of Med28 was carried out (see “Experimental Procedures”) in triplicate, and effectiveness of RNAi was confirmed by Western blot analysis (Fig. 1A). Expression profiling employing the Affymetrix mouse expression array 430 2.0 revealed 39 genes that displayed at least a 2-fold change in expression (p < 0.05) upon knockdown of Med28 (Fig. 1B and data not shown). Interestingly, among the most significant changes, we observed a striking up-regulation of SMC-related genes, including Tagln (SM22α), Actg2 (SM-γ-actin), and Cnn2 (calponin2), after Med28 suppression (Fig. 1B). Further RT-PCR and real-time RT-PCR analyses confirmed the increased expression of several SMC genes in Med28 knockdown cells (Fig. 1C, left panel). In addition, we examined NIH3T3 cells where Med28 was stably overexpressed and observed down-regulation of the respective SMC genes, confirming that regulation of SMC genes by Med28 is bona fide and specific (Fig. 1C, right panel). Western blot analyses of Med28 knockdown and overexpressed cells were consistent with RT-PCR results (Fig. 1D). Taken together, these results suggest that Med28 normally functions as a negative regulator of smooth muscle differentiation.

Med28 Suppression in C2C12 Cells Induces Transdifferentiation into Smooth Muscle Cells—To study the effect of Med28 suppression in a more relevant cell type, we examined the expression of Med28 in a primary rat vascular smooth muscle cell line as well as multipotent mesenchymal-derived murine myoblast precursors (C2C12). Since the expression of Med28 was very low in rat vascular smooth muscle cells, we chose C2C12 cells for subsequent experiments. Consistent with results obtained with NIH3T3 cells, suppression of Med28 in C2C12 cells also resulted in the up-regulation of SM22α, SM-α-actin, CRP1, and SM-γ-actin, as revealed by Western blotting and RT-PCR (Fig. 2, A and B). The SM-specific transcript encoding SM-MHC also showed an up-regulation by RT-PCR, further confirming that the expression of Med28 negatively regulates SM-specific genes (Fig. 2B). Both myogenin and MyoD, markers associated with skeletal muscle differentiation, did not increase upon Med28 knockdown (data not shown). More interestingly, C2C12 cells where Med28 was down-regulated by RNAi exhibited a distinct large and flattened cell morphology resembling smooth muscle cells (Fig. 2C, panel c) when compared with cells transfected with non-targeting control siRNA (Fig. 2C, panel a). Immunofluorescence analysis of SM-α-Actin further confirmed the presence of smooth muscle cells in Med28 knockdown C2C12 cells (Fig. 2C, panel d) when compared with control cells (Fig. 2C, panel c). Our data therefore clearly demonstrate for the first time that Med28 suppression can result in transdifferentiation of C2C12 myoblasts into smooth muscle cells. In addition, up-regulation of SM-MHC, a definitive marker of smooth muscle cells, confirms that the observed morphology following Med28 suppression is that of smooth muscle cells and not myofibroblasts.

Exogenous Expression of Med28 Rescues the SM Morphology Induced by Med28 Knockdown in C2C12 Cells—To determine whether the observed morphology changes were in fact due to down-regulation of Med28, we carried out rescue experiments by co-transfecting mouse-specific siRNA of Med28 along with
GFP-tagged hMED28. Overexpression of GFP-hMED28 partially rescued the smooth muscle morphology induced by Med28 knockdown in C2C12 cells (Fig. 2D, panels a and b). GFP-negative cells (Fig. 2D, panel d, dashed oval) clearly revealed the smooth muscle cell morphology (Fig. 2D, panel c, dashed oval; actin-phalloidin staining), whereas GFP-positive cells, representing hMED28 overexpression, displayed a wild-type morphology (Fig. 2D, panels c and d, arrows). These results further confirm that MED28 functions as a repressor of smooth muscle differentiation in these cells.

**MED28 Acts within a Submodule of the Mediator to Suppress SM Cell Fate**—Since MED28 is present both in the nucleus and in the cytosol associated with the cytoskeleton, we raised the question whether the negative regulation of SMC differentiation by Med28 is dependent or independent of its function as a mammalian Mediator subunit. Employing an anti-MED28 antibody developed in-house, first we examined the association of Med28 with representative mammalian Mediator subunits Med6, Med17, Med8, and Cdk8 in NIH3T3 cells and found that Med28 can co-immunoprecipitate at the endogenous levels with Mediator subunits of head module (Med6, Med17, Med8) and a kinase module component (Cdk8) (supplemental Fig. S1).

We next examined whether Med28 suppression influenced the cellular levels of selected Mediator complex subunits in C2C12 cells. Interestingly, we observed a consistent down-regulation of head module subunits Med6, Med17, Med8, and Med18, in Med28 knockdown cells (Fig. 3A). Levels of Med17, another head module subunit, did not change. In addition, Cdk8, a subunit of the kinase module, revealed a trend toward down-regulation (Fig. 3A). We therefore raised the question whether suppression of another Mediator subunit, down-regulated upon Med28 knockdown, also results in up-regulation of SMC differentiation markers. We chose Med6 as a representative to carry out siRNA-mediated suppression in C2C12 cells and observed that Med6 suppression, similar to Med28 sup-
pression, resulted in up-regulation of SM-α-Actin and SM22α expression (Fig. 3B) along with other SMC markers including SM-MHC (Fig. 3C). Similar to Med28 knockdown, Med6 down-regulation resulted in transdifferentiation of C2C12 myoblasts into smooth muscle cells (Fig. 3D, panels a and b). Taken together, these results suggest that Med28 functions as a negative regulator of SMC differentiation in concert with Med6 and perhaps other subunits within the head module. Our results support the idea that Med28 may function as a scaffolding protein by maintaining the stability of a submodule within the head module.

DISCUSSION

The highly conserved, multiprotein Mediator complex functions as a bridge between transcription factors bound at upstream promoter elements/enhancers and RNA polymerase II/general initiation factors at the core promoter (8–10). Mammalian Mediator is composed of at least 30 subunits and, similar to yeast Mediator, is organized into subcomplexes and modules (9, 15). MED28, which we originally identified as a merlin interactor (magicin), is established as one of the head module subunits (16, 17). Our results presented here show that knockdown of Med28 induces expression of several SMC markers including SM-MHC, and overexpression of MED28 represses SMC gene expression. More importantly, multipotent myoblasts can transdifferentiate into smooth muscle cells when Med28 is down-regulated. Furthermore, Med28 knockdown results in down-regulation of at least three other subunits in the head module as well as Cdk8, a subunit in the kinase module. A significant decrease in Med6 and Med8 in Med28 knockdown cells is suggestive of a possible physical interaction between these subunits. A similar phenomenon has been reported previously in cell extracts deficient for Drosophila Trap80, where two other subunits, dMed6 and Trfp, were down-regulated, suggesting a physical association between these subunits (18).

Our results for the first time demonstrate that a submodule within the mammalian Mediator complex could function in a highly specific manner to repress smooth muscle differentiation. Our data also suggest that MED28 may function as a scaffolding protein by maintaining the stability of this submodule, and the dosage of MED28 may be critical for this submodule to effectively repress smooth muscle differentiation. Mediator complex is believed to be required for transcription of genes involving the RNA polymerase II machinery. However, the presence of specific subunits appears to determine the regulation of distinct expression programs via interactions with relevant gene-specific transcriptional activators. The possibility of subunit composition of the Mediator complex as a determinant for tissue specificity of gene expression has also been raised (9, 19). Many Mediator subunits play a crucial role in the activation of eukaryotic mRNA synthesis through interactions with transcriptional activators, RNA polymerase II, general transcription factors, and other cofactors. However, Mediator complex also has the ability to function in transcriptional repression when associated with kinase module subunits such as MED12, MED12L, MED13, MED13L, CDK8-CycC, and CDK8L (9, 10).

The association of different kinase modules with Mediator is thought to regulate a distinct subset of genes (7, 20). A very recent report that quantitated the abundance of subunits in human Mediator showed that Mediator preparations purified through FLAG-tagged MED28 contained substantially higher levels of kinase module components and significantly less RNA polymerase II (17). Our data, demonstrating for the first time that MED28 functions as a repressor of SMC differentiation, are in concordance with this report and support the idea that the presence of the kinase module could play a role in this negative regulation. However, we cannot rule out the possibility that MED28 can switch between an active and a repressive form dictated by conformational or cellular specificity as shown in the case of CCAAT/enhancer-binding protein-β (C/EBPβ), a transcription factor, which differentially binds to transcriptionally active or inactive Mediator complexes as determined by a Ras-induced structural alteration (21). An additional possibility that cannot be ruled out is that MED28 may in fact function in an activator pathway, promoting the expression of an SMC repressor and thus indirectly acting as a repressor of SMC genes.

SMCs are very heterogeneous and arise throughout development from multiple types of progenitors. They are highly plastic and can readily switch between proliferative and differentiated states in response to extracellular cues. Abnormalities in SMC growth and differentiation are implicated in a variety of disorders such as atherosclerosis, vascular restenosis following angioplasty, hypertension, and smooth muscle tumors (22). Smooth muscle contractile genes are activated by serum-response factor, a transcription factor that binds CArG box elements within SMC gene promoters and recruits myocardin and/or myocardin-related transcription factors to stimulate transcription of SMC contractile genes (23). Myocardin-related transcription factors are localized in the cytoplasm and translocate to the nucleus in response to Rho/actin signaling (24). The presence of MED28 in the actin–cytoskeleton and in the nucleus raises the question whether nuclear–cytoplasmic translocation of MED28 could also play a role in controlling its function in SMC repression. In addition to activation, coordinated repression of SMC genes is necessary for the phenotypic switching of SMCs, and recent studies have implicated several repressor pathways (25).

Platelet-derived growth factor-BB is a potent inhibitor of SMC differentiation, and among several transcription factors, Kruppel-like factor 4 (KLF4) and phosphorylated Elk1 play a crucial role in platelet-derived growth factor-mediated SMC suppression (25, 26). Another recent study has identified PRISM (for PR domain in smooth muscle), a novel zinc finger protein, as a transcriptional repressor of smooth muscle cells (27). Our results presented here add MED28 and associated Mediator subunits to the growing list of SMC repressors and raises the question whether MED28 can either suppress the expression of myocardin family members and/or interfere with serum-response factor binding to CArG elements. Future studies are necessary to elucidate the mechanism by which MED28 functions as an SMC repressor.

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