Identification of a *Hoxc8*-regulated transcriptional network in mouse embryo fibroblast cells

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The transcription factor, *Hoxc8*, is a member of the homeobox gene family that is vital for growth and differentiation. Previously, we identified 34 genes whose expression levels were changed at least 2-fold by forced expression of *Hoxc8* in C57BL/6J mouse embryo fibroblast cells using a mouse 16,463-gene oligonucleotide microarray. In the present study, we used the combined power of microarray profiling, global Hoxc8 DNA-binding site analysis, and high-throughput chromatin immunoprecipitation assays to identify direct and biologically relevant targets of *Hoxc8 in vivo*. Here we show that 19 of the 34 responsive genes contain Hoxc8 consensus DNA-binding sequence(s) in their regulatory regions. Chromatin immunoprecipitation analysis indicated that Hoxc8-DNA interaction was detected in five of the 19 candidate genes. All of these five target genes have been implicated in oncogenesis, cell adhesion, proliferation, and apoptosis. Overall, the genes described here should aid in the understanding of global regulatory networks of *Hox* genes and to provide valuable insight into the molecular basis of *Hoxc8* in development and carcinogenesis.

cancer | chromatin immunoprecipitation | Hox protein binding sites | microarray

Homeobox proteins are transcription factors that regulate the coordinated expression of multiple genes involved in development, differentiation and malignant transformation. The homeobox is a 183-bp DNA sequence coding a 61-aa domain defined as the homeodomain that is responsible for recognizing and binding sequence-specific DNA motifs (1). Several homeobox gene families have been identified, HOX, EMX, PMX, and MSX, as well as many more divergent homeobox genes. Among these, the *Hox* genes are most intriguing for having a recognized regulatory network structure. The Hox gene family consists of 39 genes organized in four clusters on four different chromosomes. The individual clusters span ≈200-kb and contain 9–11 genes (reviewed in ref. 2). These genes are expressed in a tissue-specific, stage-related fashion and play pivotal roles in development and differentiation. In addition to their important function in embryonic development, both gain and loss of expression for a wide variety of Hox genes have been reported in malignancies of the brain, breast, colon, kidney, lung, ovary, prostate, skin, and uterus (reviewed in ref. 3). Loss of Hoxc6 expression can induce apoptosis in prostate cancer cells (4), and Hoxb13 is a repressor of hormone-activated androgen receptor inducing growth suppression of prostate cancer cells (5, 6). Hoxb7 is a key factor for a tumor-associated angiogenic switch shown to activate basic fibroblast growth factor that in turn promotes cellular proliferation (7). Specific roles for Hoxb3 and Hoxd3 in cell proliferation have been reported during hematopoesis and angiogenesis (8, 9). Additionally, Hoxa5 and Hoxa10 regulate P53 expression in human breast cancer cells (10, 11).

Hoxc8 is expressed in both the neural tube and somitic mesoderm in the prospective thorax, and is essential for mouse forelimb and skeletal development (12). *Hoxc8*-null mutant mice show neuromuscular defects in the forelimb and skeletal defects in the ribs and vertebrae of the thorax (13). Overexpression of a *Hoxc8* transgene has been shown to cause cartilage defects with an accumulation of proliferating chondrocytes and reduced maturation in skeletal elements (14). *Hoxc8* plays an essential role in cancer development. Expression of *Hoxc8* correlates with higher Gleason grades in prostate tumors, and the overexpression of *Hoxc8* can suppress androgen-dependent transcription in prostate cancer cells (15, 16). It is selectively activated in cervical cancer cells (17) and expressed only in esophageal squamous cell carcinoma tissue, but not in noncancerous mucosa (18).

It is important to study the direct functional role of the *Hox* genes in carcinogenesis and development. Recently, we identified 34 putative mouse *Hoxc8* target genes using microarray analysis (19). In the present study, we combined the power of expression profiling, computer analysis of the putative Hoxc8-DNA recognition sequence, and high-throughput chromatin immunoprecipitation (ChIP) assays to identify *Hoxc8* direct target genes. We identified five of the 34 genes as direct downstream targets for *Hoxc8 in vivo*. These are (*i*) the zinc finger protein regulator of apoptosis (Zac1), a tumor suppressor gene; (*ii* and *iii*) neural cell adhesion molecules (Ncam) and Cadherin 11 (Cdh11) supporting cell–cell and cell–matrix adhesion; (*iv*) pigment epithelium-derived factor (PEDF) playing an important role in angiogenesis; and (*v*) osteopontin (OPN) serving as a key regulator of carcinogenesis, metastasis, and skeletogenesis. These five genes are all involved in crucial biological processes such as morphogenesis, differentiation, and tumorigenesis. Our results substantially expand our knowledge of the Hoxc8 regulatory network as it pertains to development and neoplasia.

Results

Search for Hoxc8 Consensus Recognition Sequences in the 5′-Flanking Regions of Perturbed Genes. In our previous microarray study, we identified 34 genes whose expression level was changed at least 2-fold by forced expression of *Hoxc8* in C57BL/6J MEF cell lines (19). To identify genes more likely to be direct targets of *Hoxc8* regulation, we aligned the 34-gene set with mouse genomic sequences to map their 5′ sequences. Because most of the known *Hox* binding sequences are located in the proximal promoter region, the area of the upstream region between −1 and −1,000 relative to the transcription start site was retrieved. We then used the TRANSFAC match program (20) to search for *Hox* consensus recognition sequences in the upstream regions of the 34-gene set. Nineteen genes were found to contain one or more *Hox* consensus sequences in their proximal upstream region. Besides these proximal upstream regions, we also searched the *Hox* consensus recognition sequences in the 10-kb upstream regions of these 34 candidate genes and found that the Hox protein core consensus binding elements TTAT, TAAT, and TTAC, were

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Abbreviations: ChIP, chromatin immunoprecipitation; E*n*, embryonic day *n*; WISH, whole-mount *in situ* hybridization.

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present in all sequences searched. Because of the widespread distribution of *Hox* consensus binding sites in the mouse genome, the presence of binding sites is not likely to be instructive in identifying direct versus indirect targets of *Hoxc8* gene regulation. Therefore, to further identify the direct targets of *Hoxc8*, we used ChIP to detect binding of Hoxc8 to the predicted Hoxc8-binding sequences in the *Hoxc8* overexpressing MEF cell lines.

***Hoxc8* Binds to Five of the Regulated Genes in a DNA Sequence-Specific Manner.** To distinguish direct versus indirect targets of *Hoxc8* regulation, high-throughput ChIP assays were adopted. We focused on 19 genes whose proximal promoters were found to contain one or more consensus *Hox* binding sites. The ChIP assays were performed on the predicted Hoxc8 DNA-binding sites in the promoter of these 19 genes. MEF cell lines transfected with pcDNA4-Hoxc8 or pcDNA4-control were treated with formaldehyde to crosslink proteins to DNA. After sonification, the cross-linked chromatin was immunoprecipitated with Hoxc8-specific antibody. The cross-linked protein was then uncoupled from DNA by de-crosslink and proteolysis. Finally, the immunoprecipitated DNA was analyzed by quantitative PCR to determine which potential DNA-binding sites were actually bound by Hoxc8 *in vivo*. The ChIP assays were considered positive if the predicted Hoxc8-binding sequence(s) was enriched 3-fold greater than a no antibody control in three independent experiments. PCR primers were designed to generate a PCR amplicon that contained the predicted Hoxc8-binding sites. It should be emphasized that all 19 TTAT-, TAAT-, and TTAC-positive genes were subjected to ChIP analysis, and only the five indicated genes (see below) were ChIP positive, indicating the direct binding specificity.

The ChIP results (Fig. 1) demonstrate that Hoxc8 protein interacts directly with predicted Hoxc8 binding sequences in *Cdh11* (Cadherin 11); *Ncam* (Neural cell adhesion molecule); *Opn* (Osteopontin); *pedf* (pigment epithelium-derived factor) and *Zac1* (the zinc finger protein regulator of apoptosis). Hoxc8 binding sequence(s) was enriched 5.5, 4.9, 6.0, 4.1, and 5.7 times in *Cdh11*, *Ncam*, *Opn*, *pedf*, and *Zac1*, respectively.

Because MEF cell lines used in the above ChIP analysis were cultured from 15.5-days postcoitum C57BL/6J embryos, we wished to further detect whether Hoxc8 protein can bind to the promoter of these five genes at the same mouse embryonic stage. For this reason, embryonic day 15.5 (E15.5) mouse embryos were cross-linked with 1% formaldehyde diluted in serum-free DMEM for 15 min at room temperature. Quantitative PCR using the Hoxc8 antibody precipitated chromatin showed the predicted Hoxc8 binding sites were enriched 5.8, 3.4, 4.7, 3.6, and 4.3 times in *Cdh11*, *Ncam*, *Opn*, *pedf*, and *Zac1*, respectively, in the E15.5 ChIP assay (Fig. 1). The above results further verified that these five genes are authentic Hoxc8 target genes *in vivo*.

***Hoxc8* Regulates the Level of *pedf*, *Ncam*, *Zac1*, *Opn*, and *Cdh11* in MEF and HeLa Cells.** To determine whether the expression level of the five identified *Hoxc8* direct target genes are regulated by Hoxc8 protein, quantitative RT-PCR assays were performed to detect their expression differences between the MEF cell lines transfected with pcDNA4-Hoxc8 or the pcDNA4 empty vector. As shown in Fig. 2*A*, *pedf*, *Ncam*, *Zac1*, and *Opn* were down-regulated 2.4, 2.5, 2.4, and 3.3-fold, respectively when *Hoxc8* was overexpressed in MEF cell lines; in contrast, the expression of *Cdh11* was increased 2.9-fold by *Hoxc8* overexpression. Quantitative RT-PCR results are consistent with the previous microarray analysis (19). Comparison of activation of these five *Hoxc8* target genes in the control and the *Hoxc8* overexpressed NIH 3T3 cell lines demonstrated similar results with MEF cells (data not shown).

To gain further insight into the Hoxc8 regulation of *pedf*, *Ncam*, *Zac1*, *Opn*, and *Cdh11* transcription, we have cloned an ≈1-kb DNA fragment of the promoter regions of each of these five genes into a pGL3-basic luciferase reporter vector to detect their responsiveness to Hoxc8. The five luciferase reporter constructs were cotransfected in HeLa cells with pcDNA4-Hoxc8 or pcDNA4 expression vector separately. The transfection of the five constructs resulted in a significant increase in luciferase activity compared with the pGL3-basic control vector, especially *Opn*, that showed a 26-fold induction (Fig. 2*B*). In addition, Hoxc8 has an obvious effect on *pedf*, *Ncam*, *Zac1*, *Opn*, and *Cdh11* gene expression. As shown in Fig. 2*B*, an increase of Hoxc8 protein expression induces an ≈2.4-, 1.5-, 1.7-, and 1.9-fold decrease in the expression of *pedf*, *Ncam*, *Zac1*, and *Opn*, respectively, and a 2.8-fold increase in the expression of *cdh11*. Our luciferase results provide an additional line of evidence that Hoxc8 can regulate the expression of these five genes.

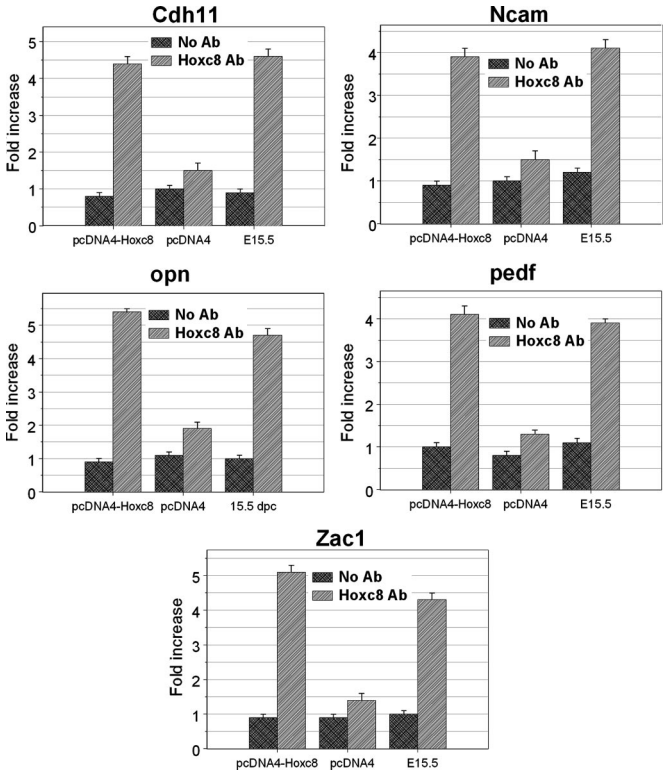

Fig. 1. Hoxc8-DNA interaction detected by ChIP assay. MEF cells transfected with *Hoxc8* expression plasmid (pcDNA4-Hoxc8) or pcDNA4 empty vector were used for ChIP assays. E15.5 mouse embryos were also used for ChIP assay. The assays were performed by using antibody recognizing Hoxc8 or no antibody as a negative control. Immunoprecipitated DNA was analyzed by quantitative PCR using Hoxc8 binding site-specific primers. *Cdh11*, *Ncam*, *opn*, *pedf*, and *Zac1* were enriched >3-fold greater than no antibody negative control. The ChIP assays were repeated in three independent experiments with similar results.

Target Gene Expression *in Vivo*. To determine whether the identified five *Hoxc8* target genes are normally expressed in embryo stages that express *Hoxc8*, and therefore present an opportunity for regulation, we performed quantitative RT-PCR on these five genes for expression in the E9.5, E10.5, E11.5, and E15.5 stage embryos (Fig. 3). As shown in Fig. 3, *Hoxc8*, *pedf*, *Ncam*, *Zac1*, and *Cdh11* express highly in all embryonic stages. Interestingly, *Opn* expresses at a low level in E9.5 and E10.5 stages, and then after E11.5 rises to relatively high levels. *Opn* mRNA levels increase ≈6-fold from E9.5 to E15.5 (Fig. 3).

After quantifying variations in the RNA levels of these five

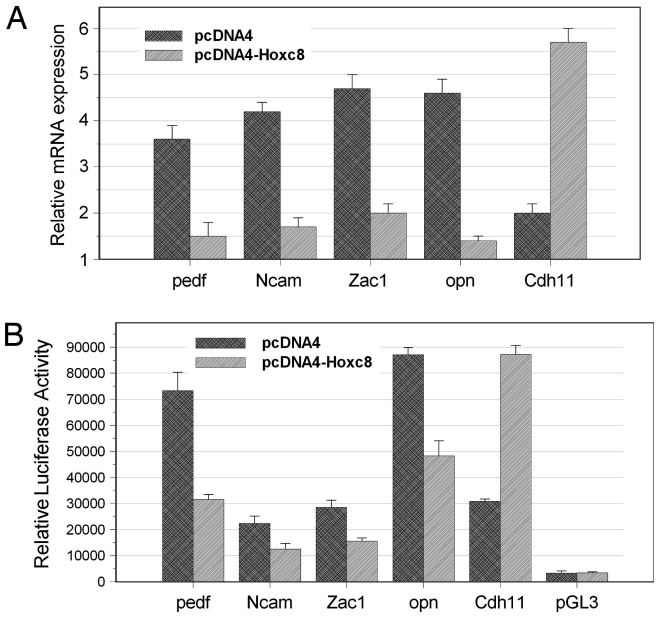

Fig. 2. Effects of Hoxc8 on *pedf*, *Ncam*, *Zac1*, *opn*, and *Cdh11* mRNA and promoter expressions in MEF and HeLa cells. (*A*) Quantitative RT-PCR analysis of *pedf*, *Ncam*, *Zac1*, *opn*, and *Cdh11* mRNA expression in MEF cells transfected with *Hoxc8* expression plasmid (pcDNA4-Hoxc8) or pcDNA4 empty vector. All results were normalized to the β-actin value. (*B*) Relative luciferase activities for different constructs. HeLa cells were plated at a density of five $\times$ 10^4 cells per well in 12-well plates for transfections. *pedf*, *Ncam*, *Zac1*, *opn*, and *Cdh11* promoter constructs (0.3 μg) were cotransfected with 0.1 μg of *Hoxc8* expression plasmid (pcDNA4-Hoxc8) or 0.1 μg of pcDNA4 empty vector separately. Transfected cells were harvested and lysed in 48 h after transfection. The luciferase activity was measured and normalized to the *Renilla* luciferase level as internal controls. Data are shown with the mean of $\pm$ SD of triplicates.

candidate genes over developmental time, we performed whole-mount *in situ* hybridization (WISH) assays using E9.5, E10.5, and E11.5 stage embryos to detect whether *Zac1*, *Opn*, and *Cdh11* have an *in vivo* expression pattern that overlaps with *Hoxc8*. *Hoxc8* expression was detected in the paraxial mesoderm, somites, and neural tube (Fig. 4*A*). High levels of *Zac1* expression was detected in brain, somites, neural tube, hindlimb, and forelimb (Fig. 4*B*). As shown in Fig. 4*C*, upon WISH staining, *Cdh11* expression is clearly observed in branchial arches, somites, forelimb, and hindlimb (Fig. 4*C*). *Opn* expression could not be detected at E10.5 along the anterior/posterior axis; however, its expression was clearly detectable in the eye, forelimb, and hindlimb at E11.5 (Fig. 4*D*).

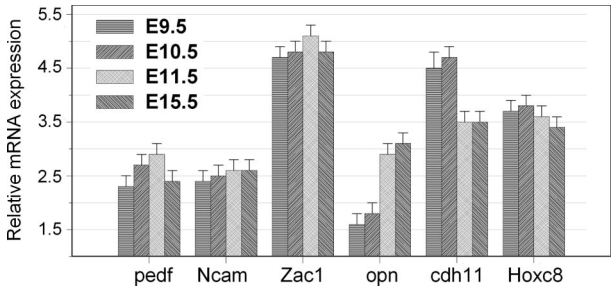

Fig. 3. *pedf*, *Ncam*, *Zac1*, *opn*, *Cdh11* and *Hoxc8* expression level at different mouse embryo stages. Quantitative RT-PCR analysis of *pedf*, *Ncam*, *Zac1*, *opn*, *Cdh11*, and *Hoxc8* mRNA expression in E9.5, E10.5, E11.5, and E15.5 mouse embryo stages. The level of gene expression was normalized to β-actin. Results are representative of three independent experiments.

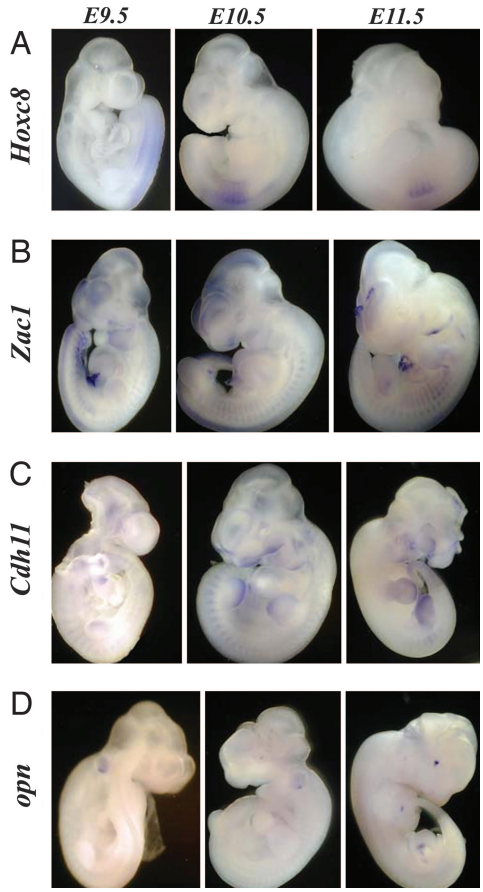

Fig. 4. *Hoxc8*, *Zac1*, *Cdh11*, and *opn* expression pattern during mouse early embryogenesis. Whole-mount *in situ* hybridization of *Hoxc8* (*A*), *Zac1* (*B*), *Cdh11* (*C*), *opn* (*D*) mRNA at E9.5, E10.5, and E11.5.

Discussion

Hox genes encode transcription factors that play a vital role in embryonic development, cell fate determination, differentiation, and proliferation. Recent reports have demonstrated differences in *Hox* gene expression between normal and neoplastic tissues, but *Hoxc8*'s role in neoplasia remains elusive. To decipher the mechanisms by which *Hox* genes regulate development and neoplasia, it is necessary to characterize their upstream regulators and downstream target genes. We have used microarray and ChIP to validate direct targets of Hoxc8, namely, *Ncam*, *Cdh11*, *Opn*, *pedf*, and *Zac1* as *Hoxc8* direct target genes. Strikingly, the function of these five potential *Hoxc8* target genes has been implicated in embryonic and cancer development. In fact, all of these five candidate genes are members of the classical FGF, BMP, WNT, p53, or NF-KB signaling pathways. The seminal regulatory roles of these five *Hoxc8* target genes (Fig. 5) are described below.

Cell Adhesion. During embryonic development, cell–cell interactions mediated by cell adhesion molecules play key roles in the control of cell movement, aggregation, and migration, and also influence cell proliferation and differentiation. Hox proteins similarly are important developmental regulators that interact in multiple germ layers to coordinate pattern formation, cell division, cell migration, and differentiation. Increasing evidence functionally links *Hox* genes to cell adhesion molecules. Here, we identify three cell adhesion molecules, *Ncam*, *Cdh11* and *Opn*, as *Hoxc8* direct target genes.

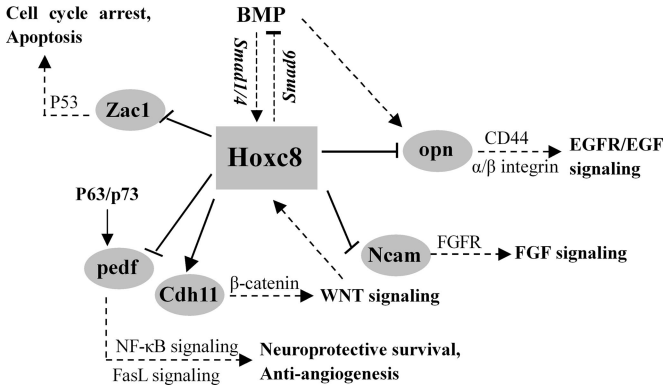

Fig. 5. The *Hoxc8*-regulated transcriptional network *in vivo*. *Hoxc8* can regulate the cross-talk between Wnt, BMP, FGF signaling pathway by direct down-regulating *Ncam*, *opn*, *pedf*, and *Zac1* and up-regulating *Cdh11 in vivo*. Direct pathways are indicated by solid lines and arrows, and indirect consequences of pathway activation and inactivation are indicated by dotted lines.

Ncam. *Ncam* is down-regulated 2.5-fold by Hoxc8 in MEF cells (Fig. 2*A*). NCAM is a Ca^{2+}-independent Ig related cell–cell adhesion molecule that mediates homotypic and heterotypic cell–cell and cell–matrix adhesion (21). *Ncam* transcripts are first detected around day 8.5 in somites and neural tube, and it is thought to play an important role as a regulator of morphogenetic processes. The regional specificity of NCAM in the neural tube added further weight to the notion that *Ncam* may be a downstream target of *Hox* gene products (22). *Hoxc6*, which displays overlapping expression with *Ncam* along the body axis induces a positive regulatory effect on the promoter of *Ncam*. *Hoxb9* induces *Ncam* transcription by binding to its promoter; however, cotransfection with *Hoxb8* antagonizes transactivation (reviewed in ref. 23). Although *Ncam* has been extensively studied as a cell–cell adhesion molecule, recent reports highlight the role of *Ncam* in signal transduction. Loss of NCAM function during tumor progression affects cell–matrix adhesion through the loss of FGFR-induced, integrin-mediated cell–matrix adhesion (24).

Cdh11. Cadherins modulate calcium ion-dependent cell–cell adhesion and are important in cell aggregation, migration, and cell sorting. Cadherin 11, or OB-cadherin, is a member of the cadherin family (25). Hoxc8 up-regulates *Cdh11* expression 2.9-fold in MEF cells (Fig. 2*A*). WISH staining shows that *cdh11* is highly expressed in the branchial arches, head mesenchyme, somites, and the distal portion of limb mesenchyme (Fig. 4*B*). The expression pattern of *Cdh11* correlates with mesenchymal morphogenesis in the head, somite, and limb buds of early mouse embryos. *Cdh11* is essential for the process of the segmentation of the paraxial mesoderm layer (26). In the trunk region of the *Hoxc8* knockout mice, several skeletal segments were transformed into the likeness of more anterior ones (13). *Cdh11*, also localized to the cell membrane, can mediate the formation of a functional adherens junction complex, recruiting β-catenin to the membrane. The expression of *Cdh11* does not dramatically alter anchorage-independent growth or cellular proliferation rate, but has been shown to produce significant changes in the invasive capacity of cancer cells (27).

OPN. OPN is a secreted, integrin-binding glycophosphoprotein that has been linked to tumor progression and survival in several solid tumors, including head and neck cancers (28). Here, we identified *Opn* as a *Hoxc8* direct target gene that is down-regulated 3.3-fold by Hoxc8 in MEF cells (Fig. 2). Previously, Shi *et al.* (29) showed that Hoxc8 binds directly to the *Opn* promoter region by gel-shift analysis assay. Our ChIP analysis supports and extends their findings. Upon WISH staining, *Opn* expresses in the eye and forelimb and hindlimb buds (Fig. 4*D*). Bone morphogenetic protein (BMP)/Smads signaling has been reported to induce *Opn* expression in a Hoxc8- regulated way (29). Currently, cumulative evidence suggests that OPN functions in the regulation of tumor metastasis and invasion. OPN is a ligand for the integrin and CD44 families of receptors, and this binding allows OPN to mediate adhesive cell–matrix interaction and to activate cellular second messengers in signal transduction pathways. It has been reported that the integrin-dependent induction of human mammary epithelial cells migration by OPN involves and depends on the activation of at least two different growth factor/receptor pathways (EGF ligands/EGFR and HGF/Met) and multiple different signal transduction pathways (30, 31).

Angiogenesis and Apoptosis. *Hox* gene expression is linked to apoptosis and angiogenesis. *Hoxa5* and *Hoxa10* can regulate p53 expression in human breast cancer cell lines (10, 11). Induction of *HOXA5* expression causes caspase 2- and caspase 8-mediated apoptotic cell death (32). *Hoxb5* and *Hoxb7* also have roles in angiogenesis (7).

pedf. Here, we show that *pedf* is a direct target of *Hoxc8* in that it was down-regulated 2.4-fold by Hoxc8 in MEF cells (Fig. 2*A*). PEDF, an angiogenesis inhibitor with neurotropic properties, balances angiogenesis in the eye and blocks tumor progression (33). However, PEDF is not simply an antiangiogenic factor: it has neuroprotective effects in the nervous system and an apoptotic effect in endothelial cells. PEDF interacts with the NF-κB pathway to stimulate the transcription of neuroprotective genes in the nervous system; and PEDF can generate anti-angiogenic and apoptosis signal by activating the Fas–Fas ligand (FasL) death cascade in endothelial and epithelial cells (34). Additionally, PEDF contributes as a natural angiogenesis inhibitor in two hormone-sensitive organs, the prostate and pancreas (35). *pedf* has also been reported to be a direct target of *p63* or *p73*, *p53* family member genes (36).

Zac1. *Zac1* is directly down-regulated 2.4-fold by Hoxc8 in MEF cell (Fig. 2*A*). Zac1 is a zinc finger transcription factor that elicits antiproliferative activity and is a potential tumor suppressor gene that exhibits its tumor suppressor activity characterized by induction of apoptosis and G_1 arrest. *Zac1* is the first gene besides *p53* that concurrently induces apoptosis and cell cycle arrest (37). As a transcription factor, *Zac1* shares a number of similar functions with *p53* (38). During mouse embryogenesis, *Zac1* was shown to have strong expression in brain areas; in addition, *Zac1* expression was detected in the neural tube, somites, forelimbs, and hindlimbs (Fig. 4*B*). The *Zac1* transcript is predominantly localized in developing chondrogenic tissue and suggested that *Zac1* is a potential regulatory gene involved in chondrogenic differentiation and cartilage development (39, 40). Overexpression of a *Hoxc8* transgenic mice has been reported to cartilage defects (14). Here, we identified *Zac1* as a *Hoxc8* target gene *in vivo*, so it is possible that Hoxc8 protein altered the chondrocyte differentiation by regulating the *Zac1* expression in the early embryo stage.

Hox genes are master transcription factors, and they function at the top of a genetic hierarchy controlling pathway. Multiple cellular processes are regulated through the *Hox* network. *Hoxc8* can regulate the cross-talk between Wnt, BMP, and FGF signaling pathways by direct targeting *Ncam*, *Cdh11*, *Opn*, *pedf*, and *Zac1 in vivo* (Fig. 5). Our results provide insights in understanding the roles of *Hox* genes in cancer and development.

Materials and Methods

Cell Culture. Cells were cultured in DMEM (GIBCO/BRL) supplemented with 10% FCS (HyClone) and 100 μg/ml penicillin–streptomycin–glutamine (GIBCO/BRL). HeLa and NIH 3T3 cells were purchased from ATCC. pcDNA4-Hoxc8 and

pcDNA4-control expressing mouse embryonic fibroblast cell lines have been described (19).

Plasmid Constructions. The plasmid pcDNA4-Hoxc8 harboring mouse *Hoxc8* has been described (19). The 1-kb promoters for the *pedf*, *Ncam*, *Zac1*, *Opn*, and *Cdh11* were amplified by PCR from mouse genomic DNA and cloned into the SmaI and BglII sites of the pGL3-basic vector (Promega) to generate a luciferase reporter constructs: pedf-luc, Ncam-luc, Zac1-luc, Opn-luc, and Cdh11-luc.

Quantitative ChIP Assay. The formaldehyde cross-link and ChIP assays of tissue culture cells were performed as described (19). We followed a protocol established by the Farnham laboratory for ChIP performed with mouse embryonic tissues (http://mcardle.oncology.wisc.edu/farnham/protocols/tissues.html). Quantitative PCR using ChIP samples was carried out by using a SYBR Green qPCR kit (New England Biolabs). The fold change in the specific binding was normalized to GAPDH and mock IP values. The primer sequences are available upon request.

RNA Isolation and Real-time PCR Quantification. Total RNA was isolated with RNeasy miniprep columns plus RNase-free DNaseI set (Qiagen). The cDNA for real-time PCR was made by using the OmniScript kit (Qiagen). Real-time PCR was performed in triplicate by using Taqman probes and the ABI prism 7900 sequence detection system (Applied Biosystems). Samples were normalized to the β-actin values.

Transfection and Luciferase Assay. HeLa cells (5×10^4 cells in 12-well plates) were transiently transfected by using Lipofectamine 2000 reagent (Invitrogen) with a total of 0.3 μg of luciferase reporter plasmid (pedf-luc, Ncam-luc, Zac1-luc, Opn-luc, and Cdh11-luc) and different expression plasmids as indicated. Luciferase activities were assayed 48 h after transfection by using the Dual Luciferase assay kit (Promega) according to the manufacturer's directions. Luciferase values shown in Fig. 2*B* are representative of transfection experiments performed in triplicate in three independent experiments.

Whole-Mount *in Situ* Hybridization. Embryos were collected from matings of FVB mice at E9.5, E10.5, and E 11.5. Embryos were staged by assigning noon of the day of vaginal plug as E0.5. *In situ* probe templates were gifts from M. Chu (*zac1*, ref. 39) and D. C. Page (*Opn* and *Cdh11*, ref. 41). *Hoxc8* probe template was from our laboratory (42). DNA template was linearized and DIG labeled antisense probes were prepared by *in vitro* transcription with SP6, T3 or T7 polymerase (Roche). WISH using embryos fixed in 4% paraformaldehyde was performed according to standard procedures.

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