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Original Research

Sox9-induced chondrogenesis in mesenchymal stem cells was mediated by ERK5 signal pathway

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Abstract: Sox9 is a member of the high-mobility-group (HMG) box protein superfamily, which is expressed predominantly among cells in mesenchymal condensations during the early development of embryonic skeletons. The extracellular-signal-regulated kinase 5 (ERK5) is one of the mitogen-activated protein kinase (MAPK) family members of protein kinases. Roles for ERK5 signaling in the regulation of chondrogenesis and adult chondrocyte homeostasis have yet to be demonstrated. In this study, we found that ERK5 could down-regulate Col2al and Sox9 expression, and this down-regulation was inhibited by MEK5β, one of ERK5 inhibitor. Furthermore, we characterized the ERK5 response with the chromatin binding profile of Sox9 in MSCs in a genome-wide manner through an analysis of ChIP-seq data. This study will help to understand the interaction between the ERK5 and Sox9, and facilitate to decipher the mechanism of chondrogenesis in mesenchymal stem cells.

Key words: Sox9, ERK5, mesenchymal stem cell, chondrogenesis, ChIP-seq

Introduction

Hyaline cartilage, the most prevalent type of skeletal articulation in the human body inside the diarthrodial joint, is a smooth and resilient connective tissue that functions as a weight-bearing and nearly frictionless gliding surface. Unfortunately, an array of etiological factors can contribute to articular hyaline cartilage damage or loss, including trauma-induced injury, age-related degenerative disease such as osteoarthritis, and a variety of diverse genetic and developmental pathologies. Moreover, due to the avascular nature of this specialized connective tissue, the endogenous repair and regeneration mechanisms of the human body are unable to counteract the destruction of the joint articular surface (1-3). Although a variety of orthopedic surgical strategies, such as microfracture, pridie drilling, autologous osteochondral and chondrocyte transplantation, endeavor to restore the surface of articular hyaline cartilage, none of the current repair methodologies elicit consistent generation of long-term replacement tissue that can endure the mechanical forces typically exerted upon articular cartilage in vivo (4). Thus, there is a critical clinical need for production of tissue-engineered or regeneration of articular hyaline cartilage that replicates native tissue both structurally and functionally.

Importantly, many of the growth factors, protein kinases, and transcription factors known to control embryonic cartilage formation also play integral roles in the regulation of mesenchymal stem cell (MSC) chondrogenesis (5-8). For example, Sox9, a member of the high-mobility-group (HMG) box protein superfamily, is expressed predominantly among cells in mesenchymal condensations during the early development of embryonic skeletons (9). Mutations in human Sox9 have been observed in patients with campomelic dysplasia that is characterized by the presence of skeletal malformation and XY sex reversal (10, 11).

The extracellular-signal-regulated kinase 5 (ERK5) pathway is one of the lesser studied members of the mitogen-activated protein kinase (MAPK) family of protein kinases. Structurally, the C-terminus of ERK5 is much larger than other MAP kinases and contains both auto-inhibitory and nuclear shuttling functions (12-15). Unlike ERK1/2, ERK5 has C-terminal transactivation domain that regulates transcriptional activation of specific targets for ERK5 (16). This structure lends itself to development of specific drug targets which may not interfere with other MAP kinases' functions. This specificity may allow for targeted therapeutics to inhibit this pathway while minimizing the inhibition of other MAP kinases that may be critical to healthy cell survival. This pathway has been implicated in cell survival, antiapoptotic signaling, angiogenesis, cell motility, differentiation and cell proliferation (17-19). ERK5 knockout mice show that the major developmental function of this kinase is related to the placental, cephalic, hindgut, and importantly, cardiovascular development (20-24). ERK5 is essential for cardiovascular development and neural differentiation. Endothelial specific ERK5 knockout mice show cardiovascular defects whereas cardiomyocyte-specific knockout mice do not, suggesting that ERK5 is required in endothelial cells (25, 26). Another function of ERK5 is the regulation of neural differentiation, revealed by a study in Xenopuslaevis (27). Bobick *et al* firstly illustrate that ERK5 pathway is an important negative regulator of adult human MPC chondrogenesis (28). Furthermore, more study is needed to determine clear association between the expression and activa-

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tion of MEK5/ERK5 and cartilage formation. Roles for MEK5-ERK5 signaling in the regulation of chondrogenesis and adult chondrocyte homeostasis have yet to be demonstrated. This understanding will help to tailor treatments to situations where abnormal activation is triggered.

ChIP-sequencing (ChIP-seq) is a method used to analyze protein interactions with DNA, determining how proteins interact with DNA to regulate gene expression. ChIP produces a library of target DNA sites bound to a protein of interest *in vivo*. Then, all the resulting ChIP-DNA fragments are sequenced simultaneously using a genome sequencer.

In this study, we found that ERK5 could down-regulate Col2al and Sox9 expression, and this down-regulation was inhibited by MEK5β, one of ERK5 inhibitor. Furthermore, we characterized the ERK5 response with the chromain binding profile of Sox9 in MSCs in a genome-wide manner through an analysis of ChIP-seq data.

Materials and Methods

MSCs culture

Bone marrow was obtained from the femoral shaft of rats. MSCs were cultured in complete culture medium consisting of αMEM (Invitrogen, Carlsbad, USA), 20% fetal bovine serum (FBS, lot-selected for rapid growth of MSCs, Atlanta Biologicals), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2mM L-glutamine (Invitrogen). All of the nucleated cells were incubated in complete medium at 37°C with 5% CO2. After 24h, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). The cells were incubated for 4 days in fresh medium, harvested with 0.25% trypsin and 1mM EDTA for 5 min at 37°C. Cells were cultured for 7 days and any non-adherent cells were removed. MSCs (characterized by their morphology and adherence) were then expanded in a monolayer and used at lower passage (passage < 2). The medium was changed every two to three days.

Lentivirus Infection

The lentiviral expression system was used to express Sox9 and ERK5 in the MSCs. The lentivirus was packed and amplified in 293T cells. After culturing for 7 days, the confluent MSCs were sub-cultured in a 6-well plate at density of 4×10^4 cells/well. On the next day, lentivirus infection was carried out in complete culture medium. Lentivirus was infected at 40 multiplicity of infection (moi). The medium was changed on the next day and then every other day thereafter.

Immunohistochemistry

The immunohistochemistry for collagen II was performed using astreptavidin-biotin complex procedure. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Sections were washed with PBS and incubated with normal rat serum in PBS for 30 min at room temperature to block nonspecific sites. The sections were then incubated overnight at 4°C with the primary antibody (all from Santa Cruz Biotechnology, including goat anti-Sox9 (1:200), rabbit anti-Col2a1 (1:100), and goat anti ERK5 (1:200). The slides were washed in PBS. The sections were then incubated with a peroxidase-conjugated secondary antibody (DAKO, Germany). After being washed twice in PBS, the sections were then treated with streptavidin-biotin-peroxidase complex and peroxidase reaction was performed using Diaminobenzidine (DAB) staining (DAKO, Germany). Light microscope investigation was performed using a Zeiss Axiophot microscope.

Real time-PCR

Total RNA was isolated using a total RNA isolation kit (Macherey-Nagel, Germany). After denaturation of total RNA at 70°C for 10 min, cDNA was synthesized with OligodT primer and reverse transcriptase (TaKaRa, Japan). Real time-PCR amplification was performed using the Taqman PCR protocol and the ABI 7300 real-time PCR system (Applied Biosystems, CA). Taqman primers and probes used for amplification were as follows: Col2a1, sense primer 5'-CCTCCGTCTACTGTCCACTGA-3', anti-sense primer 5'-ATTGGAGCCC TGGATGAGCA-3'; β-actin, sense primer 5'-TTAATTTCTGAATGGCCCAGGTCT-3', anti-sense primer 5'-ATTGGTCTCAAGTCAGTGTACAGG-3'; Sox9, sense primer 5'-CCTTCAACCTTCCTCACTACAGC-3', anti-sense primer 5'-GGTGGAG TAGAGCCCTGAGC-3'. The expression level of the mRNA was normalized by β-actin mRNA expression. Data was represented as mean ± SD (n = 3).

Western blot

Protein samples of MSCs cells were subjected to electrophoresis in 9% SDS-PAGE gels. Protein was transferred to nitrocellulose membranes (Hybond C, Amersham Biosciences) and probed with rat monoclonal antibodies against Sox9, ERK5 and Col2a1 (Santa Cruz Biotechnology, USA) at the dilution of 1:1,000. Antigen–antibody complexes were revealed using alkaline phosphatase conjugated anti-mouse secondary antibody (diluted 1:20,000, Jackson ImmunoResearch, USA) in combination with Enhanced Chemifluorescence reagent (ECF; Amersham Biosciences).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously with the following modifications (29). Briefly, there were two groups in ChIP experiments, MSCs treated with lentiviral-ERK5 (LV-ERK5) and with control lentivirus. Cross-linking was quenched by addition of glycine (125mM). Cells were then washed twice with Tris-buffered saline, resuspended in 4 ml immunoprecipitation (IP) buffer and sonicated to shear DNA using Bioruptor (Diagenode). Cell debris was removed by centrifugation and the supernatant was used. Nucleo-protein extract was incubated with anti-Sox9 antibodies (Abcam, USA) at 4°C over-night on a rotating wheel. Complex was subsequently precipitated with Dynabeads (Dynal, anti-rat) for 3h at 4°C. Elution was performed for 40min at 65°C. Sample was finally treated with RNAse A for 1 h at 37°C, and cross-link was reversed by incubation for 2h at 50°C and for 8h at 65°C in 0.5 elution buffer with 50mg Proteinase K (Eurogentec). DNA was purified by phenol-chloroform extraction and quantified by Nanodrop.

Library preparation for ChIP-seq analysis and high-throughput sequencing

DNA fragments obtained from the ChIP procedure and the input control were used for library construction and sequencing with the ChIP-Seq Sample Preparation Kit (Illumina), according to the protocol provided by the manufacturer. Data was processed with the IlluminaHiSeq 2000.

ChIP-seq data analysis

The single-ended sequence reads generated from ChIP-seq experiments were aligned to the Rnor_5.0 genome of Rattus norvegicus allowing up to 3 mismatches and up to 10 hits per read (30). After reads which were mapped to mitochondria genome were removed, the peaks were called by MACS (Model-based Analysis of ChIP-Seq, version 1.4.1) (31).The false discovery rate (FDR) cut off was set as 0.1, and only peaks that were present in both replicas were preserved, giving a total of 277 peaks. Motif discovery was performed using the sequences within±100 bp around the Sox9 summit and the default background settings, i.e., sequences randomly selected from the genome with the same GC percentage content as the target sequences. Nearest genes were assigned to peaks and Gene Ontology (GO) analyses were performed by GREAT. Genomic distributions were determined by using CEAS (Cis-regulatory Element Annotation System) (32).

Statistical analysis

The normalized density of western blot bands were determined by Image J. Statistical evaluation was conducted using T-test. *P* value less than 0.05 was considered to be statistically significant.

Results

Overexpression of Sox9 stimulated chondrogenic differentiation of MSCs

Firstly, we determined the expression of Sox9 in the MSCs through the lentiviral infection. The immunostaining showed that strong expression of Sox9 was present in the MSCs after viral infection (Fig.1A). The endogenous expression of Sox9 in the MSCs was shown in Supplementary Fig.1B. The expression of Col2a1, an important molecular marker of chondrogenesis, was up-regulated after Sox9-viral infection (Fig. 1B-C). The differential expression of Col2a1 was also confirmed by real-time PCR and Westen blotting (Fig. 1D, E), suggesting that Sox9 plays a critical role in chondrogenic differentiation of MSCs. The statistical analysis for Col2al protein expression was shown in Supplementary Figure 2A.

The ERK5 signaling pathway was an important regulator of MSCs' chondrogenesis.

Similarly, western blotting result indicated that the ERK5 expression level increased in the viral-ERK5-infected cells compared to the untreated cells or control virus-infected cells (Fig.1F). The expression of Col2al, an important molecular marker of chondrogenesis, was down-regulated when ERK5 was overexpressed

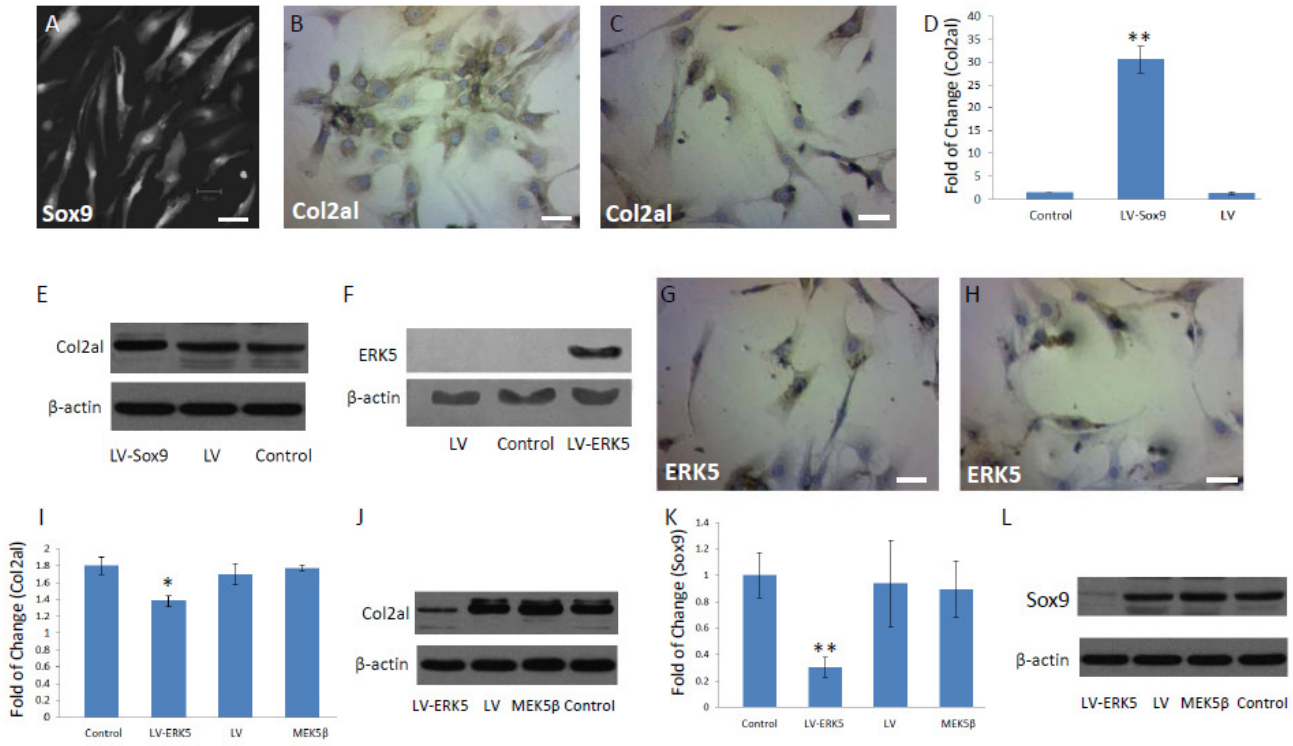
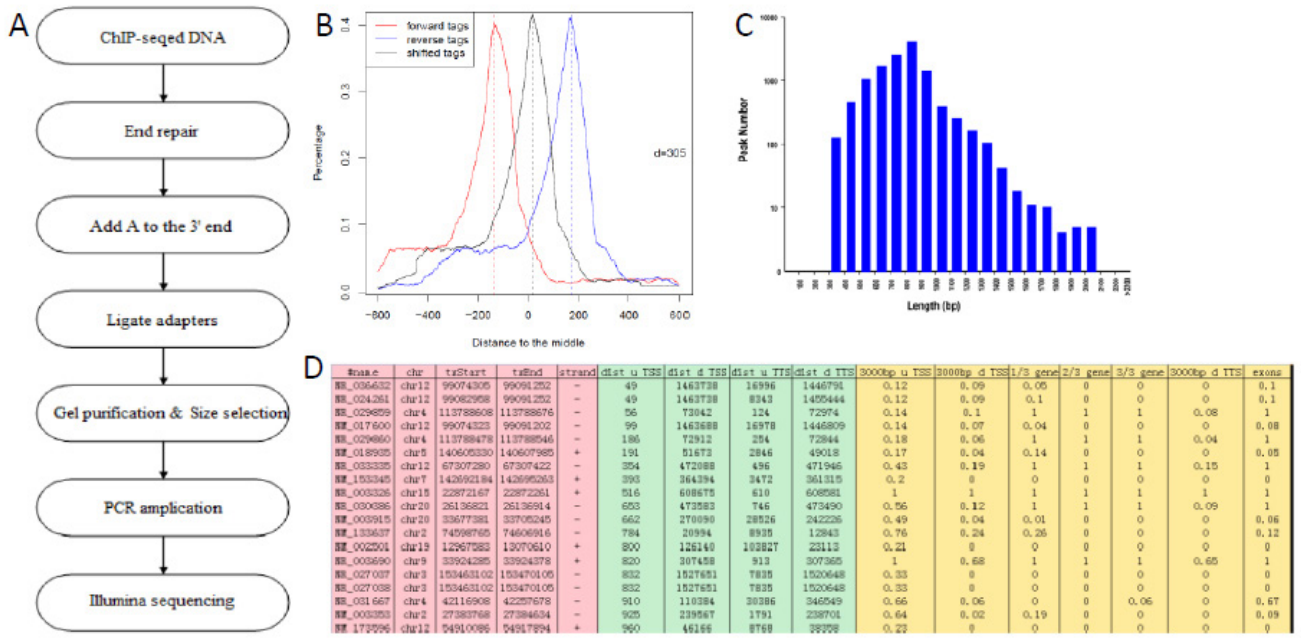


Figure 1. Chondrogenic differentiation of MSCs was regulated by Sox9 and ERK-5. (A) Sox9 expression in the MSCs through lentiviral infection. (B) Sox9 overexpression induced increaing Col2al expression in MSCs compared to that in cells infected with control lentivirus (C). (D) Increasing expression of Col2al was verified by real time PCR. **p< 0.01, determined by t-test. (E) Western blot data showed that Col2al expression level in Lentiviral-Sox9 infection group was higher than lentiviral-control infection group. (F) Western blot analysis for lentiviral-ERK5 infection in MSCs. (G) Overexpression of ERK5 down-regulated Col2al expression in MSCs compared to the control virus group (H). Decreasing expression of Col2al was verified by real time PCR (I, *p< 0.05) and Western blot (J). Application of MEK5β inhibited ERK5-induced decreasing expression of Col2al. The lower expression level of Sox9 was verified by real time PCR (K, **p< 0.01), and western blot (L). MEK5β counteracted ERK5-induced decreasing expression of Sox9. Scale bar, 10μm.



#name	chr	txStart	txEnd	strand	dist u TSS	dist d TSS	dist u TTS	dist d TTS	3000bp u TSS	3000bp d TSS	1/3 gene	2/3 gene	3/3 gene	3000bp d TTS	exons
NR_036632	chr12	99074305	99091252	-	49	1463738	16996	1446791	0.12	0.09	0.05	0	0	0	0.1
NR_024261	chr12	99082958	99091252	-	49	1463738	8343	1455444	0.12	0.09	0.1	0	0	0	0.1
NR_029859	chr4	113788608	113788676	-	56	73042	124	72974	0.14	0.1	1	1	1	0.08	1
NR_017600	chr12	99074323	99091202	-	99	1463688	16978	1446809	0.14	0.07	0.04	0	0	0	0.08
NR_029860	chr4	113788478	113788546	-	186	72912	254	72844	0.18	0.06	1	1	1	0.04	1
NR_018935	chr5	140605330	140607985	+	191	51673	2846	49018	0.17	0.04	0.14	0	0	0	0.05
NR_033335	chr12	67307280	67307422	-	354	472088	496	471946	0.43	0.19	1	1	1	0.15	1
NR_153345	chrY	14269218 4	142695263	+	393	364394	3472	361315	0.2	0	0	0	0	0	0
NR_003326	chr15	22872167	22872261	+	516	608675	610	608581	1	1	1	1	1	1	1
NR_030686	chr20	26136821	26136914	-	653	473583	746	473490	0.56	0.12	1	1	1	0.09	1
NR_003915	chr20	33677381	33705245	-	662	270090	28526	242226	0.49	0.04	0.01	0	0	0	0.06
NR_133637	chr2	74598765	74606916	-	784	20994	8935	12843	0.76	0.24	0.26	0	0	0	0.12
NR_002501	chr19	12267582	13070610	+	800	126140	102827	23113	0.21	0	0	0	0	0	0
NR_003690	chr9	339242 85	33924378	+	820	307458	913	307365	1	0.68	1	1	1	0.65	1
NR_027037	chr3	153463102	153470105	-	832	1527681	7825	1520648	0.33	0	0	0	0	0	0
NR_027038	chr3	153463102	153470105	-	832	1527681	7825	1520648	0.33	0	0	0	0	0	0
NR_031667	chr4	42116908	42257678	-	910	110384	30386	345549	0.66	0.06	0	0	0.06	0	0.67
NR_003353	chr2	27383768	27384634	-	925	239567	1791	238701	0.64	0.02	0.19	0	0	0	0.09
NR_173596	chr12	54910086	54917894	+	960	46166	8769	38358	0.23	0	0	0	0	0	0

Figure 2. (A) ChIP-seq experiment flow chart. (B) Peak model forward tags were positive match compared to the reference genome reads. Reverse tags were matches compared to the reverse complement of the reference genome reads. Shifted tags were reads after moving through the MACS model. (C) Peak length distribution analysis. (D) The list of peak related genes. The peak part was gene information on the position of the reference genomic. The green part was distance of TSS (transcription start site) and TTS (transcription termination site) between upstream and downstream of peak center. The yellow part was the ratio of peak covered parts of the gene.

(Fig. 1G, H). This down-regulation was counteracted by MEK5β, an ERK5 pathway inhibitor, using the real time-PCR (Fig.1I) and western blotting analysis (Fig. 1J). These findings suggest that ERK5 played a reverse role in chondrogenic differentiation of MSCs. The statistical analysis for Col2al protein expression was shown in Supplementary Figure 2B.

The ERK5 signaling pathway negatively regulated Sox9 expression

The finding that ERK5 pathway negatively affected chondrogenic differentiation of MSCs led us to further evaluate the influence of ERK5 on Sox9 expression, a HMG-containing transcriptional factor that plays an important role in chondrocyte differentiation and cartilage formation. As shown in Figure 1K and 1L, Sox9 expression was down-regulated at both mRNA and protein levels induced by ERK5 overexpression. This down-regulation was also recovered when MEK5βwas applied. The statistical analysis for Sox9 protein expression was shown in Supplementary Figure 2C.

ChIP-seq analysis of Sox9 binding sites in MSCs

To understand the genome-wide binding patterns of Sox9, we applied ChIP-seq technology, which is a novel approach for identifying transcription factor binding sites genome-wide. ChIP-seq flow chart was shown in Fig.2A. ChIP-seq reads generally are not protein-DNA interaction location, while they are sequences in DNA fragment 5' of ChIP. Therefore, when identifying the interaction sites, ChIP-seq reads should be moved to 3' at d/2 distance, and d is the width of the concentration peak (Fig.2B). The peak length distribution was shown in Fig.2C. The most abundant peak number was around 800-900bp size. To identify the target genes of regulated protein, we listed all the Peak related genes through CEAS software and genomic structure annotation (Fig.2D). The chromosomal distribution of ChIP region was shown in Fig.3A. Significance of ChIP enriched regions on each chromosome was indicated in bracket. Then, peak distribution was further analyzed in the four regions, including promoter, bidirectional promoter, downstream sequence of the gene and the coding region (Fig.3B). 42.9% of the peaks were on the main region, and 36.4% were on the intron. There was much less peaks in the promoter region. ChIP peaks were enriched in Chromosome 1 and 2 (Fig.3C). Distribution analysis of Peak enrichment signal on each genomic region (Fig. 4A) indicated that the signal's distance to transcription start site (TSS) was around 300bp, to transcription termination site (TTS) was around -500bp (Fig. 4B). The binding location was apparent around at the 3kb of main region, but not at the 1kb of upstream and 1kb of downstream (Fig.4C). The relative location for combined signal spectrum of exon was around 60 while there was no apparent binding site for the intron (Fig. 4D and 4E). Finally, we drew the GO annotation clustering map of peak-related gene (Fig. 4F-H). GO database applicable to all species, it can define and describe genes and proteins. We classified gene into cellular component, molecular function and biological process by GO analysis. Peak-related genomic classification on the upstream of TTS (Fig.4F), the downstream of TTS (Fig.4G) and the exon (Fig.4H) were shown. We found that after ERK5 up-regulation, there were difference in many types of cell functions related genes, such as cytoskeleton, cell proliferation, development, metabolism, and signal pathway. In addition, we also found that ERK5 may regulate many biological processes which include DNA or protein binding, and enzyme regulator activity.

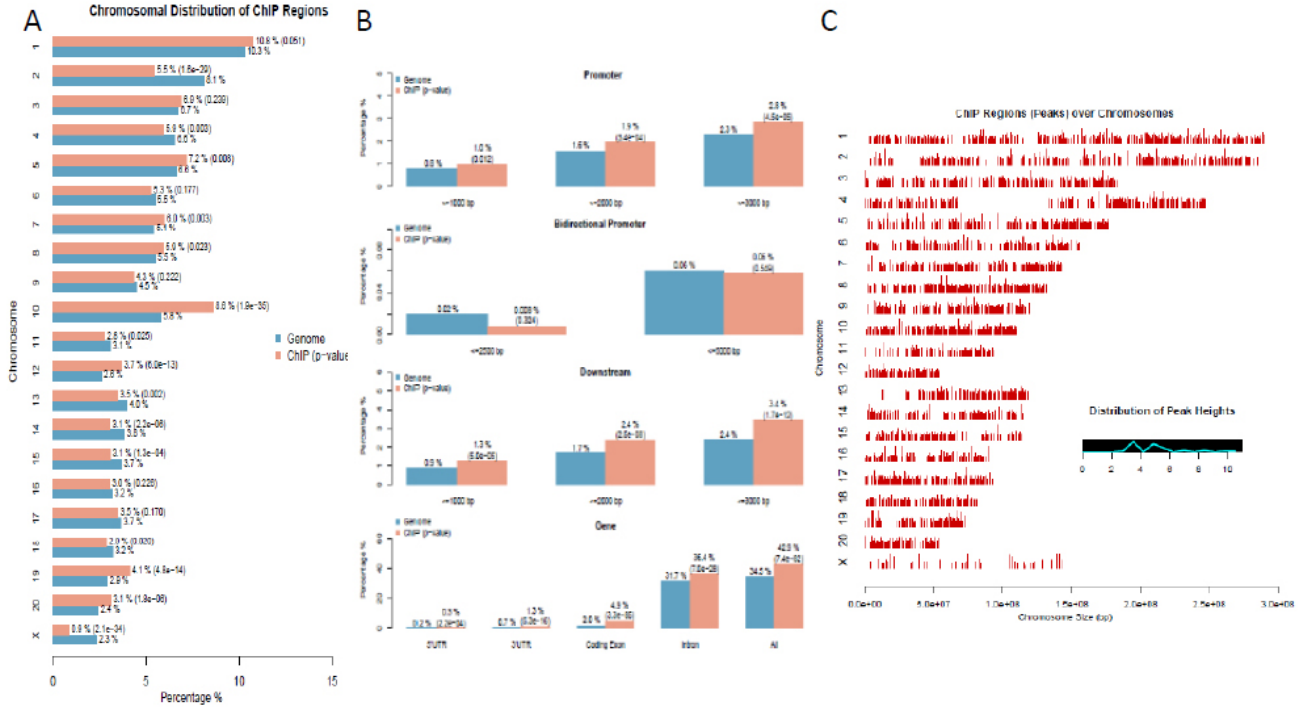


Figure 3. (A) Chromosome distribution of ChIP regions. Red bars represented the distribution of all the ChIP regions on each chromosome, while blue bars represented the distribution of target region of combined factors on each chromosome. Significance of ChIP enriched regions on each chromosome was shown in brackets. (B) Peak distribution analysis in the four regions (promoter, bidirectional promoter, downstream sequence of the gen, the gene coding region). (C) Peak enrichment signal was depicted on each chromosome. The horizontal axis represented the position on chromosome, while ordinate represented chromosome number. The red bars indicated peak enrichment signal.

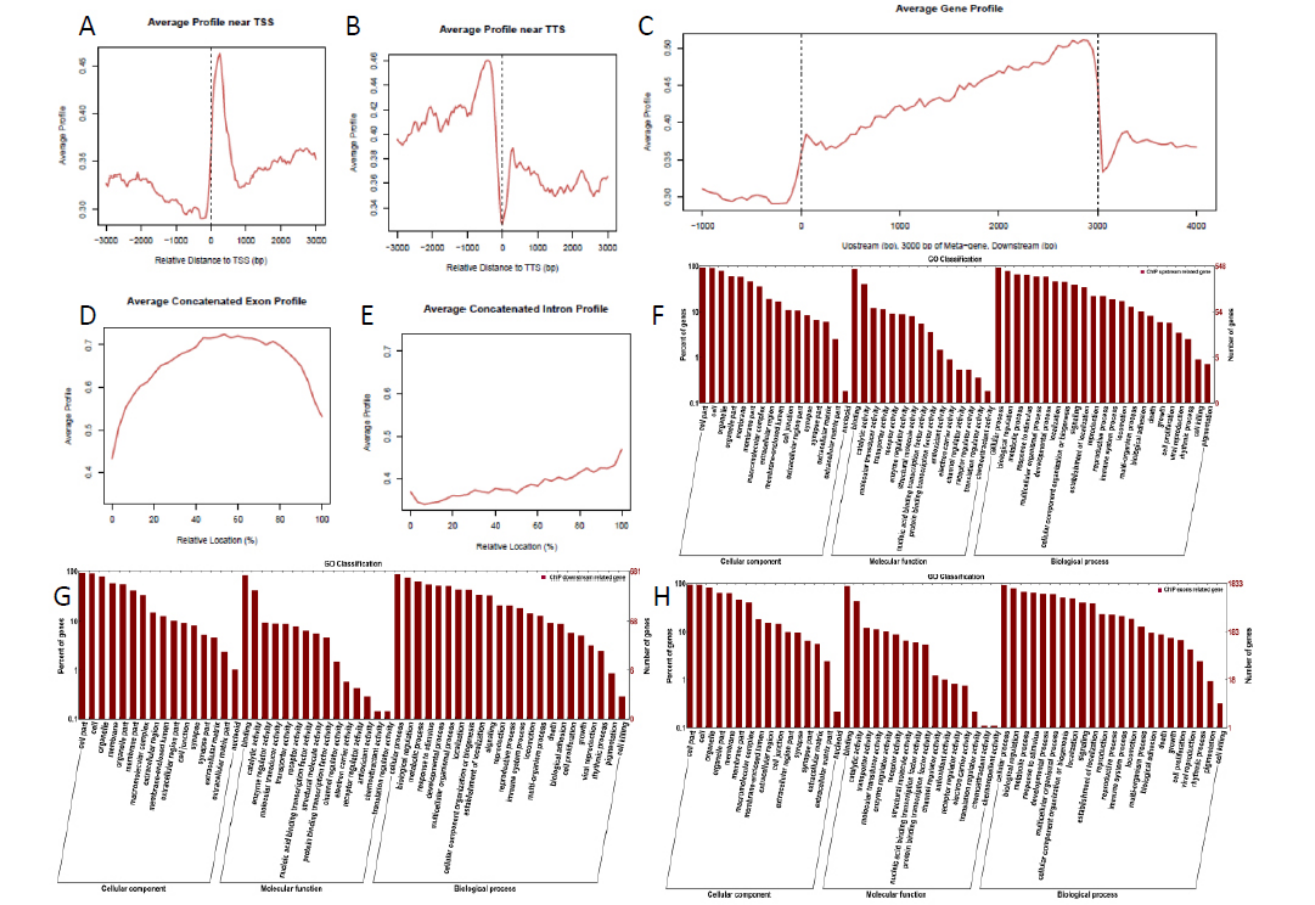


Figure 4. Distribution of peak enrichment signal on each genomic region. (A) Combined signal spectrum near TSS. (B) Combined signal spectrum near TTS. (C) Combined signal spectrums of 1kb of upstream, 3kb of main region, and 1kb of downstream. (D) Combined signal spectrum of exon. (E) Combined signal spectrum of intron. (F-H) GO annotation clustering map of peak-related gene. Peak-related genomic classification on the upstream of TTS (F), downstream of TTS (G), and the exon (H). Horizontal axis represented each segment content of GO, left vertical axis represented the percentage of genomic number and the right represented the genomic number.

Discussion

ERK5 has been reported to inhibit chondrogenesis of MSCs (33), but until now, the molecular mechanisms whereby ERK5 inhibits chondrogenenic conversion in MSCs remain largely unknown.

In our work, for the study of ERK5-inhibited chondrogenesis, we initially established the cell model system, in which ERK5 treatment resulted in a typical inhibited chondrogenic phenotype characterized by inhibition of Col2a1, Sox9, and cartilaginous ECM. Then, we found that Sox9 expression is necessary for chondrocyte differentiation of MSCs by silencing of endogenous Sox9 expression with ERK5 pathway or overexpressing exogenous Sox9.

Chondrogenesis is a complex process that is under the concerted regulation of various cytokines, growth factors, and TFs. The mechanisms whereby Sox9 contributes to chondrocyte differentiation is believed to be the results of promoting the expression of chondrocyte-specific genes, such as Col2a1 (34), Col11a2 (35), and Agc1 (36), which contain Sox9 binding sites in their promoters and serve as direct downstream targets of Sox9 transcriptional activity. However, the expression of these genes does not mean a full differentiation into chondrocyte lineage. Other signals are also required for differentiation.

We described ChIP Sequencing data obtained from mesenchymal stem cells differentiation. We demonstrated genome-wide binding pattern of ERK5-Sox9 complex that were not shown previously. In order to obtain the distribution of peak on the whole genome, we divided the rat genome into several regions (promoter, bidirectional promoter, downstream sequence of the gene, the gene main region), then we calculated the distribution ratio of genes in these four regions. These results indicate that ERK5's inhibition of Sox9, which was a transcription factor, had major role in gene regulatory regions. Less peak binding on promoter region means that Sox9 is not a target gene which ERK5 affect directly, and ERK5 may regulate the expression and transcription activity of Sox9 in other ways. Further, this dataset is part of a GO Superseries and we have used it in combination with gene expression data to associate putative target genes. To further analyze the data in an integrative manner, we conducted distribution analysis of peak enrichment signal on each genomic region, revealing the signal's distance to TSS and TTS (Fig. 4). We also provide results on binding location analysis presented in this paper that can be used for further analysis of combinatorial TF binding.

After over-expression of ERK5, we made GO annotation of Sox9 binding peak-related gene, and found that there are differential expressions of many cell functions related genes. This indicated that ERK5 has some unknown functions. To definite the regulation of ERK5 on these related-gene, it need to integrate the expression map database and make further investigation.

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