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Comparing the Middle and C-terminal Domains of the SOXE Proteins

by

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Senior Honors Project

Spring, 2017

Abstract

Osteoarthritis is the most common form of arthritis and affects millions of adults in the United States. Osteoarthritis is a degenerative joint disease that is characterized by irreversible loss of cartilage. Cartilage develops when stem cells differentiate into chondrocytes, the cells found in cartilage, through a process called chondrogenesis. Several proteins are needed for chondrogenesis, including the SOXE family of proteins. The SOXE proteins act as transcriptional activators and consist of three members: SOX8, SOX9, and SOX10. The three proteins share the same basic structure consisting of two transactivation domains. A transactivation domain is a region of a protein that can activate transcription and therefore, expression of a specific gene. In this study, I compared the activities of each transactivation domain through a dual-luciferase assay to determine the strong transactivation domain of each protein. It was found that the K2 domains of SOX8 and SOX9, and both domains (K2 and TA) of SOX10 are the strong transactivation domains. This new insight will allow further characterization of the mechanisms and functions of the SOXE proteins in the process of chondrogenesis.

Introduction

Osteoarthritis is a degenerative joint disease characterized by irreversible loss of cartilage, severe joint pain, and deformation. In 2016, it was estimated that 30 million adults in the United States suffered from osteoarthritis¹. One way osteoarthritis occurs is through improper cartilage development. Cartilage develops through a process called chondrogenesis in which mesenchymal stem cells differentiate into chondrocytes, the cells found in healthy cartilage (Figure 1).

Several proteins are involved in this process, including the SOXE family of proteins. The SOXE family of proteins consists of three members: SOX8, SOX9, and SOX10. These proteins have various roles in several developmental processes and appear to govern cell fate decisions during development by acting as transcription factors for several key proteins needed for differentiation^{2,3}. For example, the SOXE proteins have been shown to activate transcription of genes essential for sex determination and neural crest development⁶. More importantly, the SOXE proteins, particularly SOX9, are needed during the early stages of mesenchymal stem cell differentiation. SOX9 is a known transcriptional activator of the *Col2a1* gene which encodes type II collagen and the *Agc1* gene which encodes a large, very abundant proteoglycan found in cartilage⁵. Without SOX9, mesenchymal cells do not differentiate into chondroblasts, the predecessor to chondrocytes, leading to improper chondrogenesis⁵. Although not as well studied, SOX8 and SOX10 also play important roles during chondrogenesis⁵.

These proteins have similar biochemical properties and therefore, redundant functions in several processes, such as chondrogenesis². Previous research has characterized the basic structures of the three SOXE proteins. SOX8, SOX9, and SOX10 have a dimerization domain (DIM), a DNA-binding high mobility group (HMG) domain, and two transactivation domains⁶. One transactivation domain is located within the middle segment of the protein (K2) while the other transactivation domain is located at the C-terminal end of the protein (TA) (Figure 2). The SOXE proteins share a high degree of conservation within these domains. The HMG domain is the most highly conserved domain and is 79 amino acids long in each protein. SOX8 and SOX9 differ by only one residue while SOX8 and SOX10 differ by five, and SOX9 and SOX10 differ by four amino acids⁷. The DIM is also highly conserved and is unique to the SOXE family of proteins. Furthermore, there is the high degree of conservation within the K2 and TA domains.

The K2 domain shares 56 to 71 percent identity while the TA domain shares 75 to 84 percent identity between the three proteins⁷. The high degree of conservation between the characteristic DNA-binding and transactivation domains support the transcriptional activity of all three SOXE proteins. As transcriptional activators, the SOXE proteins function to initiate transcription of target genes (Figure 3). The DNA-binding domain binds to regulatory DNA sequences, and then the transactivation domains initiate transcription of the target gene. A transactivation domain is a region of a protein that can activate transcription of a specific gene and therefore, a strong transactivation domain drives a high degree of transcription while a weak transactivation domain drives transcription to a lesser amount. Even though these transactivation domains have a high degree of conservation, the activity of the domains is reportedly different in each protein. For the SOXE proteins, it is reported that K2 is the strong transactivation domain in SOX8 but not SOX9 and SOX10, whereas the TA domain is the strong transactivation domain in SOX9 and SOX10, but not SOX8⁷. Similarities in amino acid sequence and function make these differences in transactivation potentials very interesting. These findings were generated in different studies and to date, the transactivation potentials have not been directly compared in the same study. In my research, I developed a luciferase assay that allowed for the activity of all three transactivation domains to be measured at the same time under the same experimental conditions.

I followed a modified CheckMateTM Mammalian Two-Hybrid System protocol to perform the assay. In the CheckMateTM Mammalian Two-Hybrid System, a DNA-binding domain and a transcriptional activation domain are produced by separate plasmids, are transfected and expressed in the same cell, and then become closely associated thereby driving the expression of luciferase⁸. For my experiment, the pG5Luc vector contained the DNA-binding domain. The pG5Luc vector contains a Gal4 region upstream of the firefly luciferase gene.

Gal4 is a transcription activator protein with a DNA-binding domain that binds to Gal4 regions to drive gene expression. In other words, when the DNA-binding domain of Gal4 binds to the Gal4 region on the pG5Luc vector, luciferase will be expressed. The second vector, pBind, contained the *Gal4* gene and a gene that encoded the K2 or TA domain of one of the SOXE proteins. When the pG5Luc vector and the various pBind vectors are both transfected into the same cells, Gal4 and the K2 or TA domain of one of the SOXE proteins will interact, and Gal4 will drive the expression of luciferase by binding to the Gal4 region of pG5Luc (Figure 4)⁸. The first two examples in Figure 4 show the negative controls that serve to determine the background level of luciferase expression. The last example in Figure 4 demonstrates how the transcriptional activity of the K2 or TA domain of each SOXE protein will be determined. When Gal4 and the K2 or TA domain of one of the SOXE proteins interact, the expression of luciferase will be affected. If the segment is a strong transactivation domain, the expression of luciferase will significantly increase whereas the expression of luciferase will remain the same as the background level if the segment is a weak transactivation domain. Through this assay, I will be able to study the transactivation potential of each domain to better understand the mechanisms of the SOXE proteins and gain valuable insight into potential therapeutic targets for osteoarthritis.

Methods

Construction of GAL4-SOXE-pBind expression plasmids

To construct plasmids encoding fusion proteins for the GAL4 DNA-binding domain and the K2 or TA domain of the SOXE proteins, the K2 or TA domain of each SOXE protein was amplified from cDNA of each gene through PCR. Different sets of forward and reverse primers were created to amplify the specific region of interest of *SOX8*, *SOX9*, and *SOX10*:

Table 1. List of Primers

SOX8K2 Forward Primer	5'-CG <u>GG ATC CGT</u> AGT GAC CAC CAC ACA GGC CAG A-3'
SOX8K2 Reverse Primer	5'-CGG <u>AGA TCT</u> CA TGT GGG CAG GGC TGA GTG GC-3'
SOX8TA Forward Primer	5'-CG <u>GG ATC CGT</u> GGA CCC CTT CGG CCA CAG ATC A-3'
SOX8TA Reverse Primer	5'-CGG <u>AGA TCT</u> CA GGG TCG GGT CAG GGT GGT GT-3'
SOX9K2 Forward Primer	5'-CG <u>GG ATC CGT</u> CCC GGC GAG CAC TCG GGG CAA-3'
SOX9K2 Reverse Primer	5'-CGG <u>AGA TCT</u> CA CGG CAC CCC CGG GTG GCC GT-3'
SOX9TA Forward Primer	5'-CG <u>GG ATC CGT</u> CAG TCC CAG CGA ACG CAC ATC A-3'
SOX9TA Reverse Primer	5'-CGG <u>AGA TCT</u> CA AGG TCG AGT GAG CTG TGT GTA-3'
SOX10K2 Forward Primer	5'-CG <u>GG ATC CGT</u> CCA GAG CAC CCC TCA GGC CAG A-3'
SOX10K2 Reverse Primer	5'-CGG <u>AGA TCT</u> CA CAC ATG GCC TGG GTG CCC GT-3'
SOX10TA Forward Primer	5'-CG <u>GG ATC CGT</u> GTG GAT GCC AAA GCC CAG GTG A-3'
SOX10TA Reverse Primer	5'-CGG <u>AGA TCT</u> CA AGG TCG GGA TAG AGT CGT ATA-3'

Each forward primer contains a *Bam*HI site and each reverse primer contains a *Bgl*II site to facilitate restriction enzyme cloning. Primers, Agilent Technologies 10x Pfu Buffer, and Agilent Technologies Pfu Turbo (2.5 Units/uL) were used to amplify the K2 and TA domains of *SOX8*, *SOX9*, and *SOX10* by PCR. Amplicons were analyzed by gel electrophoresis using the Invitrogen SYBR Safe DNA gel stain (ThermoFisher Scientific) and bands of the expected size were cut out from the gel using a scalpel. Extracted DNA molecules were purified using the QIAquick Gel Extraction kit (Qiagen, Inc.) and were again verified through gel electrophoresis, visualizing bands with Invitrogen ethidium bromide (ThermoFisher Scientific). Before ligation, amplicons were digested with *Bam*HI and *Bgl*II which created identical sticky ends. The Promega pBind vector was digested with *Bam*HI and then dephosphorylated using New England Biolabs Calf Intestinal Alkaline Phosphatase (CIP). Digested products were then analyzed using the Invitrogen SYBR Safe DNA gel stain (ThermoFisher Scientific), cut out of the gel using a scalpel, and purified using the QIAquick Gel Extraction kit (Qiagen, Inc.). Gel electrophoresis was then done to verify the purity and determine the relative concentration of the digested

products. The K2 and TA amplicons were subsequently ligated into pBind using Roche T4 DNA ligase (Sigma-Aldrich). The ligation products were then transformed into DH10 β competent *E. coli*. DNA from resulting colonies was purified through Qiagen mini-prep kits and then analyzed by *Bam*HI and *Xba*I double digest to confirm the presence of an insert in the correct orientation. Sanger sequencing was then performed to verify positive clones. Sequenced plasmid DNA was compared to wild-type sequences using NCBI BLAST to confirm the presence of the K2 or TA domain of *SOX8*, *SOX9*, and *SOX10*, and the absence of any PCR-induced errors. Midi-preps of the verified samples were then prepared using the Qiagen Midiprep kit. The purified DNA from the midi-preps was then checked through restriction digest using *Bam*HI and *Xba*I, and subsequently analyzed by gel electrophoresis. Once confirmed by gel electrophoresis, concentration of the purified DNA was measured using a nanodrop spectrophotometer. Through this process, the K2 and TA domain of each SOXE protein was successfully cloned into a pBind vector downstream of the Gal4 DNA-binding domain.

Transfection

These plasmids were then transiently transfected into human embryonic cells (HEK293) in triplicate. The cells were maintained in cultures of DMEM that contained 10% FBS and this cell line was chosen due to its ease of transfection. Transient transfections were done using FuGENE 6 Transfection Reagent, a multi-component lipid-based transfection reagent that complexes with and transports DNA into cells during transfection (Roche). HEK293 cells were passaged one day before transfection to ensure that they were actively growing the day of transfection. The next day, the number of cells present was counted using a hemocytometer and the appropriate dilution was created to ensure that 3×10^5 cells were plated into each well.

Because 6-well plates were used, a total of 2 mL of regular culture medium was added to each well. Cells were then transfected between 4 and 6 hours after plating. While cells were attaching to the well, DNA:FuGENE 6 mixtures were created. Each mixture contained FuGENE 6, one of the pBind vectors (expression plasmids), the pG5Luc vector (reporter plasmid), and the pSV2bGal vector (control plasmid). The pSV2bGal vector serves as an internal control to normalize transfection efficiency. Because the transfection was done in triplicate, each mixture was prepared for triplicate dishes. Each mixture contained 9 uL FuGENE 6, 291 uL plain Dulbecco's Modified Eagle Medium (DMEM) culture medium (ThermoFisher Scientific), 600 ng pBind, 1200 ng pG5Luc, and 600 ng pSV2bGal. The mixtures were gently mixed and allowed to incubate for 1 hour. Then 100 uL of DNA:FuGENE mixture was added dropwise to each culture well. Plates were swirled to ensure even dispersal and placed back into the 37°C incubator for 48 hours. After transfection, each experimental well contained 200 ng pBind, 400 ng pG5Luc, and 200 ng pSV2bGal. In addition to experimental transfections, one well was transfected with 100 ng GFP and 900 ng of empty vector. Empty vector was transfected to ensure that equal amounts of DNA were transfected between all conditions. This condition was a control to verify that transfection took place.

Cell Lysis

Two days post-transfection, cell extracts were prepared. To complement the Tropix Lysis solution (Applied Biosystems), dithiothreitol (DTT) was added to a final concentration of 0.5 mM. Culture medium was aspirated out and cells were washed with ice cold 1x PBS. Then the Lysis buffer/0.5 mM DTT solution was added to each well. Cells were detached using a cell scraper, and then the buffer/cell solution was pipetted into a microfuge tube and placed on ice.

Tubes were centrifuged for 5 mins at 13,000 g at 4°C. The supernatant was subsequently placed into a new tube and stored at -80°C.

Luciferase Assay

After cells were lysed, the dual-luciferase assay was performed. Before luciferase expression was measured, Dual-Light Reagent Buffer A and Dual-Light Reagent Buffer B had to be prepared (Applied Biosystems). Buffer A was reconstituted in 5 mL sterile water, and 0.5 uL Tropix Galacton-Plus Substrate (Applied Biosystems) was added to 49.5 uL Buffer B and placed on ice. Then in a white 96-well plate, 12.5 uL of Buffer A and 5 uL of each lysate were added. Immediately after, 50 uL of Buffer B/Galacton Plus Substrate mix was added to each well and luciferase was measured for 1 second on the Wallac 1420 luminometer. Once all samples were read, the plate was wrapped in aluminum foil and allowed to incubate at room temperature in the dark for 1 hour. After an hour, 50 uL of Tropix Accelerator II (Applied Biosystems) was added to each well and light emission from the β -galactosidase reaction was measured for 1 second on the Wallac 1420 luminometer. Since light emission from the β -galactosidase reactions were expected to be the same for each sample, it was used to normalize the expression of luciferase.

Results

Regions encoding the K2 and TA domains of each SOXE protein were amplified by PCR

The K2 and TA domains of each SOXE protein were amplified from cDNA of each gene through PCR. Amplicons were analyzed by gel electrophoresis, and bands of the expected size were extracted and purified. To confirm that an amplicon of the expected size was extracted, 5uL of purified samples were analyzed by gel electrophoresis. The expected sizes for the K2 domains of SOX8, SOX9, and SOX 10 are 285 bp, 273 bp, and 270 bp, respectively. The expected sizes for the TA domains of SOX8, SOX9, and SOX10 are 384 bp, 378 bp, and 372 bp,

respectively. Gel electrophoresis indicated that PCR was successful and segments of the expected size were extracted and purified for both domains of all three genes (Figure 5A-B). The lanes were clean and only had one band, suggesting that there was no contamination and only DNA of the expected segment was extracted and purified. The first round of PCR and extraction to purify the K2 domain of SOX8 was unsuccessful (Figure 5A, lane 2) and therefore, PCR extraction was repeated and the purified amplicon was analyzed (Figure 5B, lane 2).

PCR amplicons were digested to prepare for ligation

Amplicons were prepared for ligation by restriction digest. The pBind vector was also prepared for ligation by restriction digest. To ensure that digestion was complete, amplicons and the pBind vector were analyzed by gel electrophoresis, bands of the expected size were extracted and purified. Once again, 5 uL of purified products were analyzed by gel electrophoresis. Gel electrophoresis indicated that restriction digestion and extraction were successful. Bands were of the expected size and lanes were clean, indicating no contamination (Figure 6). Additionally, the band corresponding to the pBind vector was about 10 times more intense than the bands corresponding to the K2 and TA domains of each *SOXE* gene (Figure 6). The relative concentration of these products was needed to determine the amount of each to use for the ligation reactions. The digested and purified K2 and TA amplicons were subsequently ligated into the pBind vector. Although data corresponding to the digested amplicon for SOX8 K2 is not shown, the digested amplicon was not contaminated and was the expected size.

Ligated products were transformed and plasmid DNA was purified through mini-prep

After the ligation reactions, ligated products were transformed and plasmid DNA from resulting colonies was purified through mini-preps. Analysis by gel electrophoresis of correctly oriented inserts that had been double digested with *Bam*HI and *Xba*I followed. A band of ~6500

bp representing the pBind vector was seen in each lane (Figure 7A-F). The smaller sized bands seen in some of the lanes are the inserts corresponding to the K2 or TA segment of SOX8, SOX9, or SOX10. At least one sample for each domain has a band of the expected size (Figure 7A-F). Using the results from this analysis, samples were selected to be sequenced. Sanger sequencing of positive clones (SOX8 K2 – 6, SOX8 TA – 5, SOX9 K2 – 1, SOX9 TA – 1, SOX10 K2 – 6, SOX10 TA – 2) confirmed the presence of the K2 or TA domain of *SOX8*, *SOX9*, and *SOX10*, and the absence of any PCR-induced errors.

Verified plasmid DNA was amplified and purified through midi-prep

Midi-preps of verified samples were done to amplify and purify desired plasmid DNA. After plasmid DNA was isolated through midi-prep, it was digested. Digested, purified plasmid DNA was subsequently analyzed by gel electrophoresis (Figure 8A-C). The expected sizes for the K2 domains of SOX8, SOX9, and SOX 10 are 303 bp, 291 bp, and 288 bp, respectively. The expected sizes for the TA domains of SOX8, SOX9, and SOX10 are 402 bp, 396 bp, and 390 bp, respectively. A band of ~6500 bp representing the pBind vector was seen in each lane (Figure 8A-C). The smaller sized bands were the inserts corresponding to the K2 or TA segment of SOX8, SOX9, or SOX10 (Figure 8A-C). All inserts were of the expected size indicating that the desired plasmid DNA was amplified and purified through midi-preps. Additionally, the lanes were clean and only have two bands suggesting that there was no contamination. Once confirmed by gel electrophoresis, concentration of the purified DNA was measured using a nanodrop spectrophotometer (Table 2).

Table 2. Spectrophotometric analysis of purified, digested samples

Sample	Nucleic Acid Concentration (ng/uL)	A260/A280
SOX8 K2	1923.8	1.89
SOX8 TA	2462.3	1.9
SOX9 K2	1554.1	1.88
SOX9 TA	3062.5	1.89
SOX10 K2	2635.1	1.9
SOX10 TA	1124.4	1.88

DNA concentration of the digested purified samples ranged from 1124.4 ng/uL to 3062.5 ng/uL.

Additionally, all samples had an A260/A280 ratio of approximately 1.8 suggesting pure double-stranded DNA in each sample (Table 2).

Dual luciferase-assay

The plasmid DNA constructs were then used to determine the transactivation potential of the K2 and TA domain of each SOXE protein by transfecting into HEK 293 cells. HEK 293 cells were transfected in triplicate with pG5Luc, pBind containing sequences for either K2 or TA of SOX8, and pSV2bGal (a control plasmid to normalize transfection efficiency). pBind alone was used as a negative control while SOX11, a known transcriptional activator and member of the SOXC family, was used as a positive control. The K2 domain of SOX8 showed a statistically significant increase in luciferase expression compared to the control while the TA domain showed no substantial increase in luciferase expression compared to the control (Figure 9A). This suggests that the K2 domain of SOX8 is the strong transactivation domain. The K2 and TA domains of SOX9 both showed statistically significant increases in luciferase expression compared to the control indicating that both domains act as transcriptional activators (Figure 9B). Similarly, the K2 and TA domains of SOX10 both showed statistically significant increases in luciferase expression compared to the control (Figure 9C). Luciferase expression levels were

comparable for both domains suggesting they have similar levels of activity, and both domains can activate transcription (Figure 9C).

Discussion and Conclusion

The results for SOX8 suggest that the K2 domain is a strong transcriptional activator while the TA domain is a weak transcriptional activator. Pfeifer, et al. previously reported that the transcriptional activation activity of SOX8 was due to the K2 transactivation domain³. The present results are consistent with prior work indicating that the K2 domain of SOX8 is the strong transactivation domain.

The transcriptional activation activity of SOX9 was previously studied in HeLa cells by Südbek, et al. Their research indicated that the TA domain was the strong transactivation domain while the K2 domain had almost no transactivation activity⁹. In contrast, I observed that the K2 domain of SOX9 had a higher transactivation potential than the TA domain. In fact, the TA domain of SOX9 had much less activity while the K2 domain of SOX9 had much more activity than previously reported⁹. It is possible that the two domains interact, and are both needed for the strong transcriptional activation seen *in vivo*. Further studies will need to be conducted to test this theory.

Pusch, et al. studied the transactivation potential of the K2 and TA domains of SOX10 in HeLa cells and reported that the TA domain was the strong transactivation domain¹⁰. The results of my study, which suggest that activity of the two domains is similar, somewhat disagree with the previous research. The K2 domain had a 4 fold increase of activity, and the TA domain had similar activity to what had been published by Pusch, et al¹⁰. Once again, more research will need to be conducted to further study the transactivation potentials of the SOX10 domains.

Additionally, the activity of transactivation domains of SOX8, SOX9, and SOX10 will need to be measured in chondrocyte-specific cell lines. My study utilized HEK 293 cells while previous studies worked in HeLa cells⁸⁻¹⁰. Even though both cells are easy to transfect and are immortal, HeLa cells are cancerous. Although unlikely, differences between my results and previous studies could be due to their use of a cancerous cell line. Furthermore, the SOXE proteins are needed during the early stages of differentiation during proper chondrogenesis and therefore, will likely be most active in cells differentiating into chondrocytes. Because of this, the transactivation potential of the K2 and TA domains of the SOXE proteins would be most accurate when measured in chondroprogenitor or chondroblast specific cell lines, such as RCS or ATDC5 cells.

This research has provided new insight into how the SOXE proteins function as transcription factors. Since this research expanded previous work, the new data will allow further characterization of the mechanisms and functions of the SOXE proteins in the process of chondrogenesis. Exploring and understanding the mechanisms of the SOXE proteins during chondrogenesis will contribute to the effort of finding new treatment options for osteoarthritis. For example, understanding the role transcription factors play during chondrogenesis could allow researchers to one day determine a way to reprogram an arthritis patient's mesenchymal stem cells so that they make their own healthy cartilage again. Even though there have been some improvements in treatment options, osteoarthritis remains relatively untreatable and therefore, research must be continued to find new ways of treating and preventing this condition.

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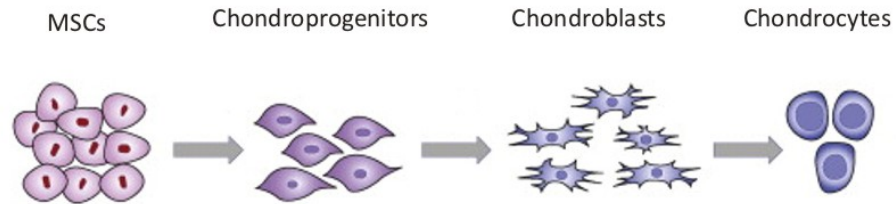


Figure 1. Overview of chondrogenesis

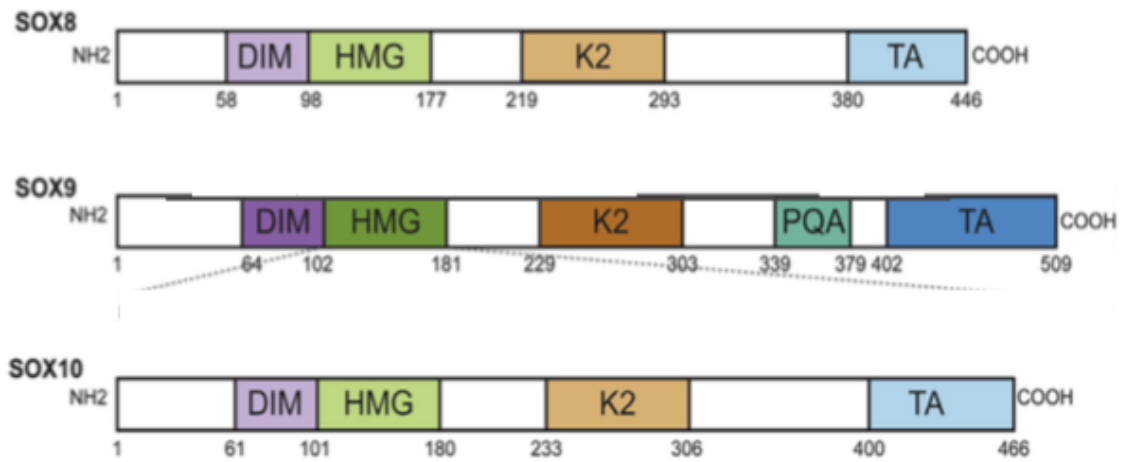


Figure 2. Homology of SOX8, SOX9, and SOX10. Abbreviations: DIM – dimerization domain, HMG - DNA-binding high mobility group, PQA – undefined domain, K2 – middle transactivation domain, TA – C-terminal transactivation domain

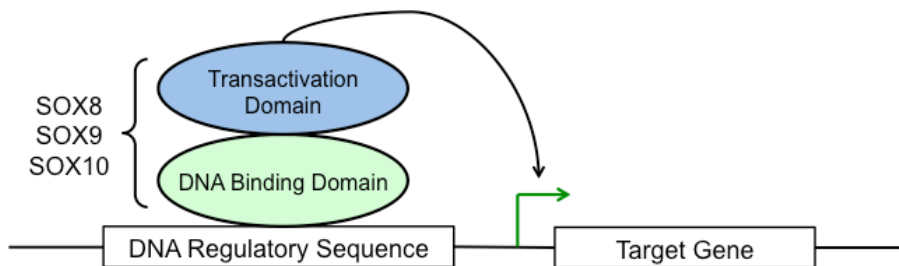


Figure 3. Transactivation domain of SOX8, SOX9, and SOX10 activate transcription of several genes important to chondrogenesis

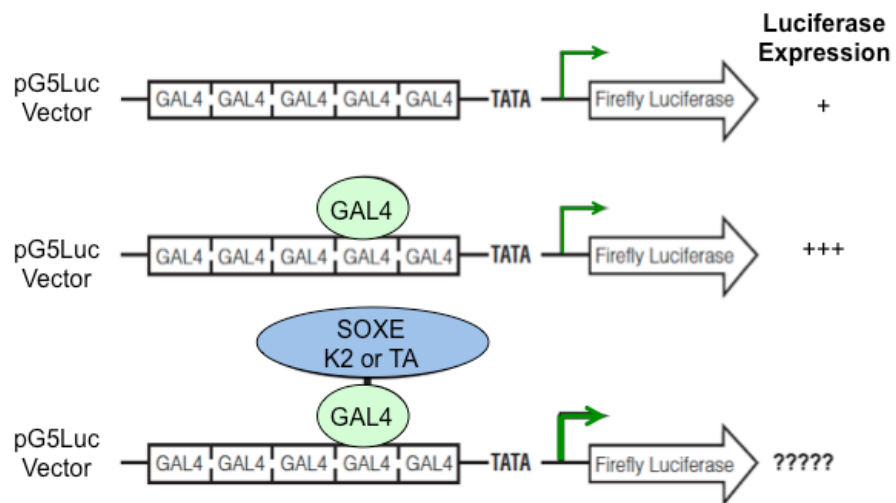


Figure 4. Modified overview of the Gal4-Luciferase Assay

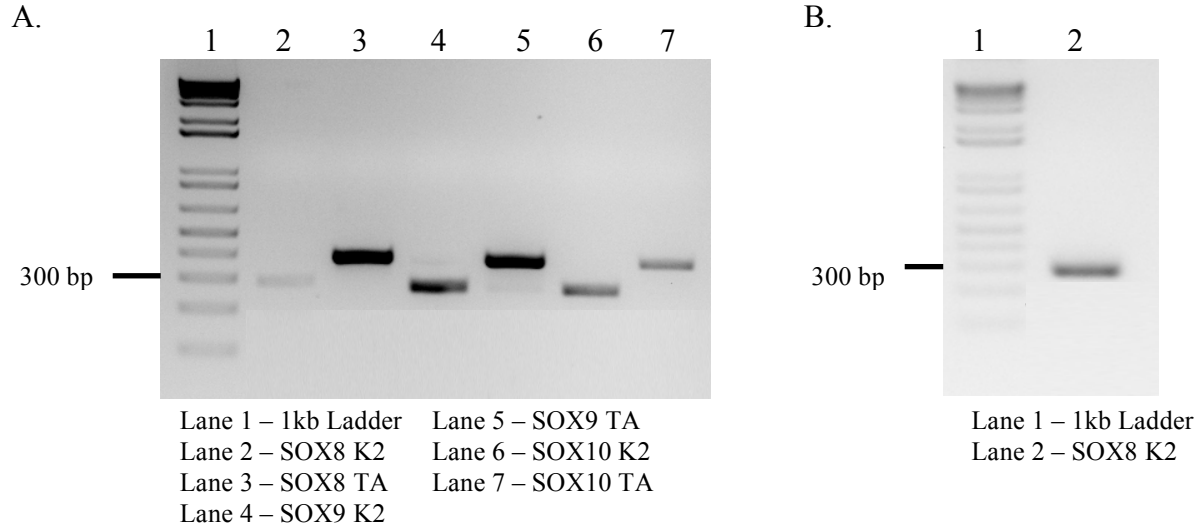


Figure 5. Analysis of extracted PCR amplicons. Specific forward and reverse primers were used to amplify segments encoding the K2 or TA domain of each *SOXE* gene. Amplicons were extracted, purified, and then analyzed by gel electrophoresis. A) The first round of PCR extraction successfully purified DNA encoding SOX8 TA, SOX9 K2, SOX9 TA, SOX10 K2, and SOX10 TA. B) The second round of PCR extraction successfully purified DNA encoding SOX8 K2.

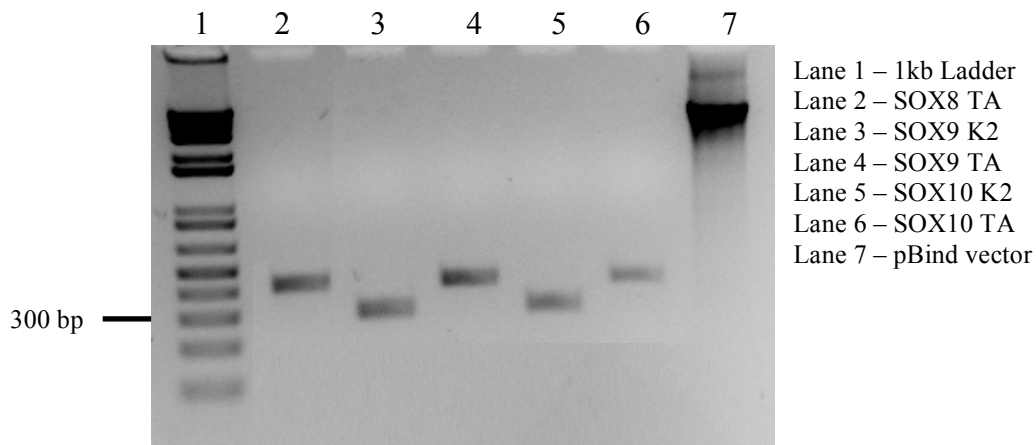


Figure 6. Analysis of extracted digested amplicons and pBind vector. After restriction digestion, products were extracted, purified, and then analyzed through gel electrophoresis. The extracted products indicate that the K2 segment of SOX9, and SOX10; the TA segment of SOX8, SOX9, and SOX10; and pBind were successfully digested, extracted, and purified. Data for the K2 segment of SOX8 is not shown.

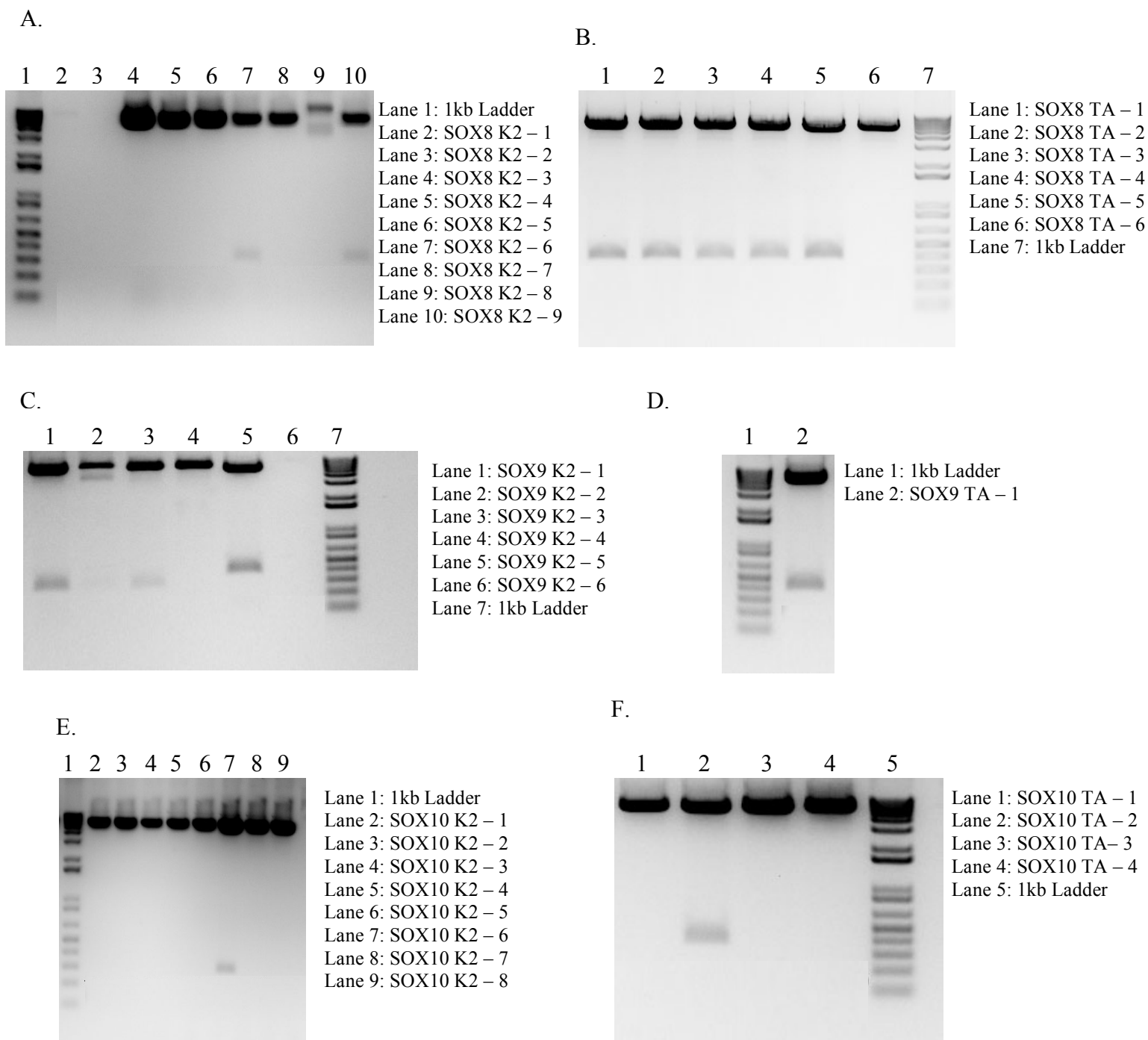


Figure 7. Analysis of purified digested plasmid DNA. After purifying plasmid DNA through mini-prep, presence of an insert was confirmed by restriction digestion. A) Digests of mini-preps of putative clones containing DNA encoding the K2 domain of SOX8 – lanes 7 and 10 have a band that corresponds to the SOX8 K2. B) Digests of mini-preps of putative clones containing DNA encoding the TA domain of SOX8 – lanes 1 through 5 have a band that corresponds to the TA SOX8 insert. C) Digests of mini-preps of putative clones containing DNA encoding the K2 domain of SOX9 – lanes 1 and 3 have a band that corresponds to the SOX9 K2 insert. D) Digests of mini-preps of putative clones containing DNA encoding the TA domain of SOX9 – lane 2 has a band that corresponds to the SOX9 TA insert. E) Digests of mini-preps of putative clones containing DNA encoding the K2 domain of SOX10 – lane 7 has a band that corresponds to the SOX10 K2 insert. F) Digests of mini-preps of putative clones containing DNA encoding the TA domain of SOX10 – lane 2 has a band that corresponds to the SOX10 TA insert. All other lanes do not have a band corresponding to an insert indicating that the colony did not contain the desired plasmid DNA.

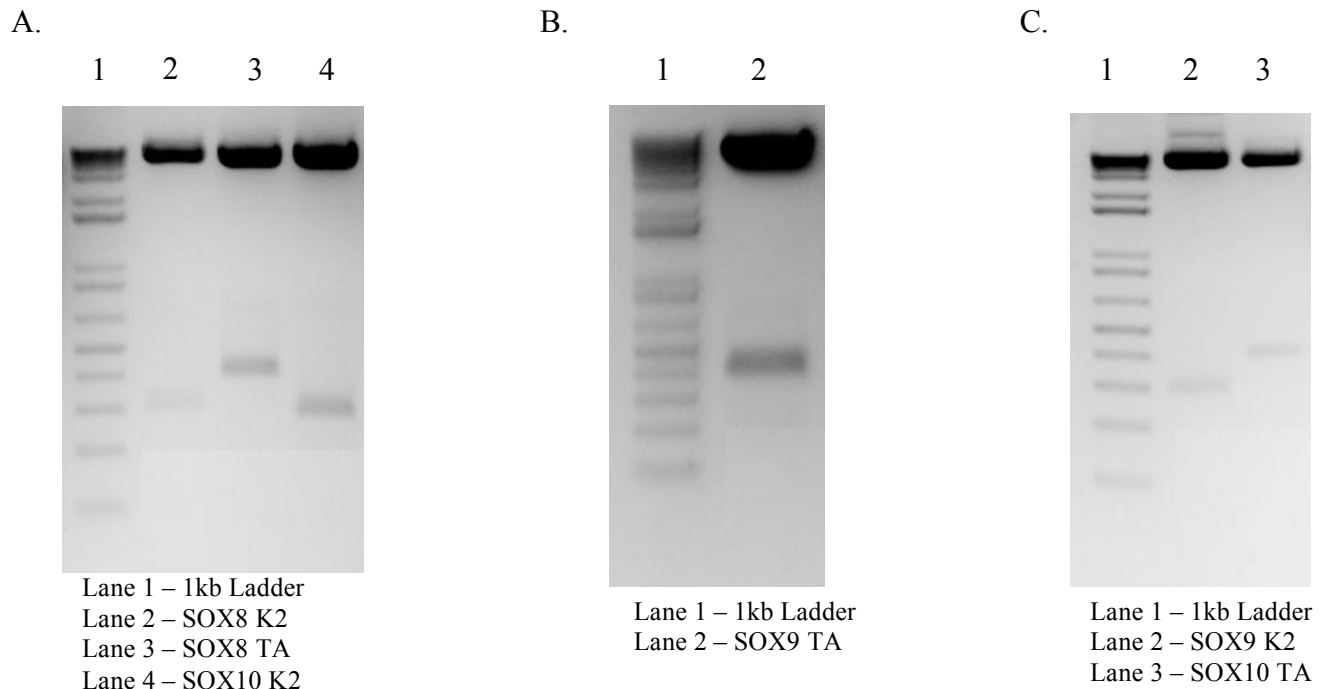
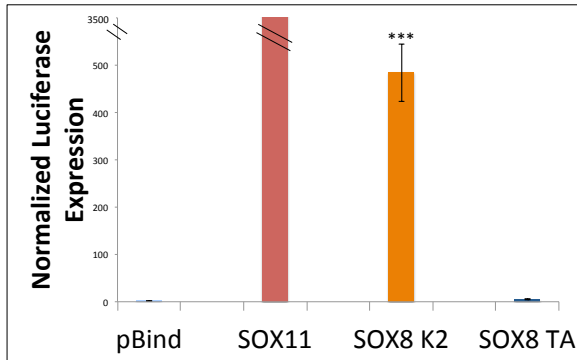
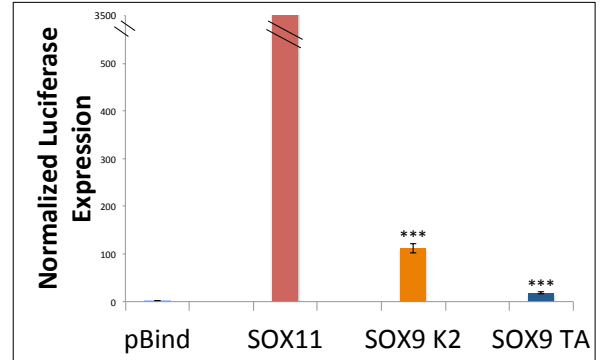


Figure 8. *Analysis of amplified, purified, and digested plasmid DNA.* After amplifying and purifying plasmid DNA through midi-prep, presence of an insert was confirmed by restriction digestion. A) Digests of midi-preps of clones containing DNA encoding the K2 domain of SOX8, the TA domain of SOX8 and SOX10. B) Digests of midi-preps of a clone containing DNA encoding the TA domain of SOX9. C) Digests of midi-preps of clones containing DNA encoding the K2 domain of SOX9 and the TA domain of SOX10.

A.



B.



C.

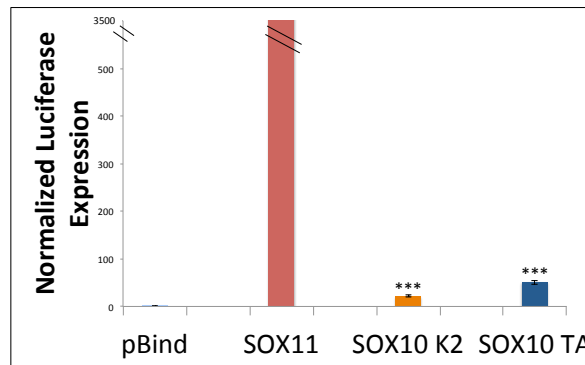


Figure 9. Analysis of transactivation potential through a dual-luciferase assay. HEK 293 cells were transfected in triplicate. SOX11, a known transcriptional activator was used as a positive control and pBind alone was used as a negative control. A) Transactivation potential of SOX8 K2 and TA domains. B) Transactivation potential of SOX9 K2 and TA domains. C) Transactivation potential of SOX10 K2 and TA domains. A-C) Each error bar represents mean \pm standard deviation of triplicate determinations. *** indicates $p < 0.001$ determined by an unpaired t-test compared to pBind.