RESEARCH ARTICLE

Porcine SOX9 Gene Expression Is Influenced by an 18bp Indel in the 5′-Untranslated Region

Bertram Brenig1☯*, Yanyu Duan2☯, Yuyun Xing2, Nengshui Ding2, Lusheng Huang2, Ekkehard Schütz1

1 Institute of Veterinary Medicine, Georg-August-University, Burckhardtweg 2, D-37077, Göttingen, Germany, 2 Key Laboratory for Animal Biotechnology of Jiangxi Province and the Ministry of Agriculture of China, Jiangxi Agricultural University, 330045, Nanchang, China

☯ These authors contributed equally to this work.
* bbrenig@gwdg.de

Abstract

Sex determining region Y-box 9 (SOX9) is an important regulator of sex and skeletal development and is expressed in a variety of embryonal and adult tissues. Loss or gain of function resulting from mutations within the coding region or chromosomal aberrations of the SOX9 locus lead to a plethora of detrimental phenotypes in humans and animals. One of these phenotypes is the so-called male-to-female or female-to-male sex-reversal which has been observed in several mammals including pig, dog, cat, goat, horse, and deer. In 38,XX sex-reversal French Large White pigs, a genome-wide association study suggested SOX9 as the causal gene, although no functional mutations were identified in affected animals. However, besides others an 18bp indel had been detected in the 5′-untranslated region of the SOX9 gene by comparing affected animals and controls. We have identified the same indel (Δ18) between position +247bp and +266bp downstream the transcription start site of the porcine SOX9 gene in four other pig breeds; i.e., German Large White, Laiwu Black, Bamei, and Erhualian. These animals have been genotyped in an attempt to identify candidate genes for porcine inguinal and/or scrotal hernia. Because the 18bp segment in the wild type 5′-UTR harbours a highly conserved cAMP-response element (CRE) half-site, we analysed its role in SOX9 expression in vitro. Competition and immunodepletion electromobility shift assays demonstrate that the CRE half-site is specifically recognized by CREB. Both binding of CREB to the wild type as well as the absence of the CRE half-site in Δ18 reduced expression efficiency in HEK293T, PK–15, and ATDC5 cells significantly. Transfection experiments of wild type and Δ18 SOX9 promoter luciferase constructs show a significant reduction of RNA and protein levels depending on the presence or absence of the 18bp segment. Hence, the data presented here demonstrate that the 18bp indel in the porcine SOX9 5′-UTR is of functional importance and may therefore indeed be a causative variation in SOX9 associated traits.
Introduction

Sry (sex determining region Y)-box 9 (SOX9) belongs to the SoxE subgroup of Sox family proteins and is expressed during embryonal development and adult life in meso-, ecto- and endo-derm derived tissues [1]. It is involved in numerous cellular processes, e.g. chondrogenesis [2], sex determination [3], pigmentation [4], organ maintenance [1], limb development [5], and cancer [6]. In the mesoderm SOX9 is involved in chondrogenesis and skeletal development [7], male gonad development, as well as development of cardiac valves and septa as well as epithelial differentiation in the pyloric sphincter [8]. However, SOX9 does not only play an important role in embryonal development. Recent data demonstrate that it is also important in the maintenance of adult organs and expression of SOX9 can be detected in adult stem and progenitor cells [9]. In mice it was shown that SOX5, SOX9, and SOX13 are expressed in adult Leydig cells and may therefore contribute to steroido- and spermatogenesis in postnatal testes [10]. In rats SOX9 expression was detected in the adult testicular cords and seminiferous tubuli suggesting a role in further germ cell differentiation [11]. SOX9 expression was also detected in goat testis in postnatal development, however, expression levels decreased to less than 50% of the concentration measured at two months of age [12].

Despite high levels of expression in both chondrogenic tissue and gonads, SOX9 is also transcribed to varying degrees in other tissues, including human adolescent heart, brain, kidney, muscle, colon, and cranial neural crest [13]. This suggests that SOX9 has other crucial functions not only in chondrogenesis and sex determination. For example, SOX9 supports tumor growth and invasion, regulates CEACAM1 expression in colon epithelium and plays a role in cranial neural crest development [14, 15].

Because of its wide range of interactions and functions, it is not surprising that mutations of the SOX9 gene locus are causative for a variety of defects in humans including campomelic dysplasia with or without sex reversal [16, 17], Pierre Robin sequence [18], Cook’s syndrome [19], 46,XY gonadal dysgenesis [20], 46,XX male sex reversal, and congenital generalized hypertrichosis with or without hyperplasia [21]. Male-to-female or female-to-male sex reversal has also been described in animals [22–26]. In a Sry-negative XX European roe deer three SOX9 copies were detected leading to an incomplete male-determination. The sex reversal was presumably due to a dosage effect. In earlier studies of canine XX sex reversal SOX9 was initially excluded as candidate gene, however, recent reports show a SOX9 duplication resulting in an overexpression [25, 27]. In pigs female-to-male sex reversal of 38,XX animals has been described in several studies and it was shown that SOX9 expression is elevated in XX sex-reversed or intersex gonads [23, 24]. Recently, a genome-wide association study performed in the French Large White population demonstrated that the only significantly associated SNPs clustered around the SOX9 locus [28]. Comparative sequencing of the candidate region in affected animals and controls revealed 14 different polymorphisms. Unfortunately, all of these were located outside of the exons or splice-sites and therefore were questioned as functional candidate mutations [28]. However, at least three haplotypes were deduced that were more frequently present in the affected animals. These haplotypes included polymorphisms located in important regulatory regions as well as the 5’- and 3’-UTR of SOX9. One of the regulatory regions is the so-called TES (testis specific enhancer) approx. 13 kb upstream the transcription start site. It was shown in mice that within this region a highly conserved core element TESCO is bound by different transcription factors resulting in either up or down regulation of SOX9 [3, 29]. But SOX9 expression regulation is complex and under the control of further distant located elements. For example, eight (E1–E8) evolutionary conserved elements have been identified by comparative analysis. Five of these elements are dispersed in a region 290 kbp upstream and three up to 452 kbp downstream of SOX9. Two further elements were localized in the 3’-UTR of SOX9 [30].
recent experiments using chromosome conformation capture-on-chip analysis even more distant regulatory regions influencing SOX9 expression have been identified [31]. These regions are located 2.46 Mb upstream as well as 1.22 Mb and 1.6 Mb downstream of SOX9. SOX9 also regulates its own expression in a positive feedback mechanism by the so-called SOM, an enhancer identified in mice 70 kb upstream of Sox9 [32]. In this respect it is noteworthy that a potential SOX9 consensus binding site [33] can be found approx. 17.8 kb upstream the porcine SOX9 transcription start site. Besides the long-range regulation, SOX9 expression is, of course, also regulated by promoter elements. In the murine Sox9 promoter an interval between -193 and -73 was identified to be essential for maximal promoter activity and tissue-specific expression in mouse cell lines [34]. Subsequently, several studies reported that functional elements in this region, i.e. two functional CCAAT boxes, a cAMP-response element (CRE) half-site and two specificity protein 1 (SP1) binding sites within 150 bp upstream the transcription start site (TSS), regulated SOX9 transcription in mouse and human chondrogenic cell lines and primary chondrocytes [35, 36]. In humans it was shown that SOX9 expression is regulated by Sp1, CREB, and CBF [37, 38]. These binding sites are located in the proximal promoter upstream the transcription start site. In vitro mutagenesis of these sites results in a reduction of SOX9 promoter activity. To define the regulatory network in response to different hormones and cellular signals, it is important to characterize the cis-regulatory elements even in the minimal promoter region. Functional elements located between -256 and +67 are important for controlling human SOX9 transcription efficiency [35]. However, transcription elements in the interval between -73 and +251 determine the minimal transcriptional activity of the mouse Sox9 promoter [34]. Whether additional regulatory elements are influencing SOX9 transcription downstream the transcription start site has not been shown so far.

In this context, it is noteworthy that in the French Large White population mentioned above, an 18bp indel was detected in the 5' UTR in three of the sequenced control animals [28]. In this contribution we report that the same 18bp indel is also present in other pig breeds, i.e. German Large White, Laiwu Black, Bamei, and Erhualian. The occurrence of the 18bp indel in such diverse pig breeds in conjunction with the results reported about the porcine 38,XX sex reversal phenotype prompted us to address the question whether this region has an influence on SOX9 expression and might therefore at least partly explain the sex reversal. This hypothesis is further supported by the fact that the 18bp indel region harbours a CRE half-site which is usually recognized by the cAMP-response element binding protein CREB [39].

**Materials and Methods**

**Animals**

Seven Chinese domestic pig breeds (Erhualian, Laiwu Black, Bamei, Wuzhishan, Hang, Jianhexiang White, Tibetan) and different Western commercial pig breeds (Pietrian, German Landrace, German Large White, Red Duroc) were investigated. Blood samples were drawn by veterinarians as part of routine diagnostic procedures (parentage control, epidemiological testing) with informed owner consent, therefore the study was exempt of ethical approval according to the German regulations. A total of 938 animals were used for the identification of polymorphisms in the SOX9 gene. The complete SOX9 gene including 2 kb up- and downstream sequences was analysed.

**PCR fragment length polymorphism (PFLP) analysis**

PFLP analysis was used to screen all samples using the following PCR primers: forward 5’-GCCAGTTTTACCCCCAGGA-3’ and reverse 5’-AGCGGCTCCCGGCAA GCCT-3’. The amplicon has a length of 189bp and 207bp for the Δ18 and wild type alleles, respectively. PCR
was performed in a final 20 μL reaction mix contained 40 ng DNA, 1 x PCR buffer, 2.0 mM MgCl₂, 0.2 mM of each dNTP, 10% DMSO, 10 μmol of each primer and 1 unit of ExTaq DNA polymerase (Takara). The PCR profile was as follows: 3 min at 94°C; 35 cycles of 30 s at 94°C and 1 min at 68°C; and a final extension of 7 min at 72°C. PCR products were evaluated by electrophoresis on 2% agarose gels.

Tissue culture

The cell lines used for the experiments were selected based on their reported transfection efficiencies and level of SOX9 expression. Nuclear extract from ESK-4 cells (female embryonal porcine kidney) were used for the immunodepletion EMSA in order to keep within the autologous porcine system. In addition it was shown previously that SOX9 is expressed in the embryonal kidney and therefore the presence of essential regulatory factors was assumed [40]. No data on SOX9 expression in PK-15 are available. Expression of SOX9 has been shown in ATDC5 [41]. In HEK293T SOX9 was not detectable using antibody NBP1-85551 (Novus Biologicals). However, due to a lower transfection efficiency of porcine epithelial PK-15 and mouse pre-chondrogenic ATDC5 cell lines, we also used human kidney HEK293T, which is known to have a higher transfection efficiency [41-44].

HEK293T, PK-15, and ATDC5 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM glutamine at 37°C in a humidified atmosphere containing 5% CO₂. Porcine kidney ESK-4 cells were purchased from the European Collection of Cell Cultures (ECACC) and cultured according to the manufacturer’s recommended protocols.

Affinity purification of CREB

DNA-binding affinity was analysed as described [45] using three oligonucleotides containing either the CRE half-site (WT: 5’-CGCTGCAGCCGAGTGACGCGCCAG GCTTCC-3’), a mutated CRE half-sitemut (MT: 5’-CGCTGCAGCCGAGCTCTACGCCAGGCTTCC-3’) or the consensus CRE binding site (CRE: 5’-AGAGGATTGCCT GACGTCAGAGAGCTAG-3’). HEK293T cells were transfected using HA-tag CREB-expression plasmid (pcDNA4-HA-CREB, see below) and nuclear and cytoplasmic extracts were prepared after 32 h. Extracts were incubated with the biotinylated probes (WT, MUT, CRE) and Streptavidin MagneSphere Paramagnetic particles (Promega) for 2 h at room temperature. The reactions were separated using a MagneSphere Magnetic Separation Stand (Promega) and washed according to the manufacturer’s instructions. Eluted extracts were separated on SDS/PAGE and analysed by Western blotting using a phospho-CREB antibody (Millipore).

Electromobility shift assay (EMSA)

Nuclear and cytoplasmic extracts were prepared from sub-confluent ESK-4 cells by NE-PER Nuclear and Cytoplasmic Extraction Kit (PIERCE) according to the manufacturer’s instructions. The DNA-protein interaction was studied by EMSA following the instructions of Chemiluminescent Nucleic Acid Detection Module (PIERCE) with minor modifications as previously described [46]. Briefly, binding reactions included 2 μL 10 x binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), 5% BSA, 0.5 mM EDTA, 5% glycerol, 1 mM MgCl₂, 100 ng poly-dA-dT, 0.1% NP-40 and 4-4.5 μg nuclear and cytoplasmic proteins. Binding reactions were pre-incubated for 20 min on ice followed by 20 min at room temperature after adding 20 fmol biotin-end labelled double strand oligonucleotide probe (wild type: 5’-gcgccttcctaaagctgcgctgcgcagccttcccgagcgagcc-3’; Δ18: gcgccttcctaaagctgcgctgcgcaggcttcccgagcgagcc-3’). Finally, the mixtures
were loaded onto 7% acrylamide–0.5x Tris-borate-EDTA gel (acrylamide/bisacrylamide = 37.5:1). For competition analysis, 2–5 pmol unlabelled competitors, i.e. the 18-bp fragment, were added to the binding reactions. For interrogation of the binding transcription factor, 3 μL phospho-CREB antibody (Millipore) or E2F4 antibody (Millipore) was pre-incubated with nuclear and cytoplasmic proteins on ice for 2 h to get the maximal signal. The mix was added to the above binding reactions for 20 min on ice prior to the addition of the labelled DNA probe.

**Plasmid constructs**

To analyse the effect on expression of CREB binding to the wild type 5′-UTR pcDNA4-HA-CREB, pGL4.20-WT (wild type) and pGL4.20-Δ18 (mutant) were constructed. Full-length human CREB cDNA (Sino Biological Inc.) was subcloned into the pcDNA4-HA expression vector. pGL4.20-WT and pGL4.20-Δ18 was generated by subcloning the porcine SOX9 promoter between position -100 and +399 (TSS = +1) into the KpnI/HindIII sites of the pGL4.20 vector. The promoter was obtained by artificial gene synthesis (Sangon).

To determine the effect of Δ18 on translation, two plasmids (psiCHECK-2-WT-TTG and psiCHECK-2-Δ18-TTG) were constructed by inserting the 5′-UTR plus the start codon of SOX9 into an Nhel site upstream the Renilla luciferase gene. Before inserting the DNA fragments into the psiCHECK–2 vector, the start codon of the Renilla luciferase was mutated to TTG by PCR-driven overlap extension. By this way, the Renilla luciferase translation would be driven by the start codon of SOX9. The 5′-UTR of SOX9 was PCR amplified using the primers: forward 5′-TTTTGCTAGCAAGAGCCCCTGGGCTGGGAGTTGG-3′ and reverse 5′-TTTTGCTAGCAGGCAGGGAGGGGACGCAGGCCCAGGGAAC-3′. Amplified products were cloned and ligated into the Nhel site directly preceding the Renilla luciferase gene in the plasmid psiCHECK-2-TTG. The psiCHECK2-TTG vectors are ideal for examining the effect of 5′-UTRs on gene expression as described [47, 48]. By fusing the 5′-UTR of interest to the TTG mutated Renilla luciferase gene, luciferase activity can be used as a marker for 5′-UTR regulation.

**Luciferase reporter assays**

Cells were seeded at 2 x 10^5 cells per well in 24-well dishes. When the cells were 80% confluent, the luciferase constructs were transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Thirty-six hours after transfection, cells were harvested and assayed for luciferase activity using a dual-luciferase reporter assay kit (Promega) following the manufacturer’s manual. Results are given as relative light units (RLU) that is Firefly luciferase value/Renilla luciferase value. The average RLU of cells co-transfected with pGL4.20 and pRL-TK (Renilla luciferase control vector, Promega) was considered to be 1. In each experiment 0.2 μg of pGL4.20, pGL4.20-WT, or pGL4.20-Δ18 was added together with 50 ng pRL-TK without or with 0.8 μg pcDNA4-HA-CREB. The optimal amount and ratio of reporter and control plasmids have been determined in preliminary experiments. The amount of pRL-TK was adjusted according to previous reports [49–51].

Student’s t-test was used to calculate significance (* p<0.05, ** p<0.01, *** p<0.001). All experiments were performed in triplicate.

**Real-time quantitative PCR assays**

Total RNA was extracted with the TRIZOL Reagent (Invitrogen) according to the manufacturer’s instructions. 6 μg of RNA was transcribed into first-strand cDNA using Superscript First-Strand Synthesis System for RT-qPCR (Takara). The relative expression levels were analysed using Brilliant II FAST SYBR QPCR Master Mix (Stratagene) in an MX3000P system (ABgene)
according to the manufacturer’s instructions. The following primers were used: Renilla 1, 5′–CTGATCTGATCCGGATGTGTT–3′ and 5′–GACGATGCCCTTGATCTTG–3′; ß-actin 1, 5′–AAGGAGAAG CTGTGCTACGTCG–3′ and 5′–TGAAGGTAGTTCTCTGGATGCC–3′.

Statistical analysis
Putative transcription factor binding sites within Δ18 were analysed with MatInspector (http://www.genomatrix.de/matinspector) using default settings. The DNA sequence between the transcription start site and the start codon was used as input. The promoter alignment analysis of evolutionary relationship among pig and other vertebrates was performed using ClustalW2 (http://www.ebi.ac.uk/). Expression data were analysed using GraphPad Prism™ 5.0 for Macs (GraphPad Software, San Diego, CA).

All transfection experiments were performed in at least three independent experiments using three biological replicates and standard errors (SEM) were calculated using Microsoft Excel for Mac 2011 (version 14.5.4).

Results
CREB binds to the CRE half-site within the 5′-UTR of the porcine SOX9 gene and influences expression
In an analysis of 938 animals of different commercial and Chinese pig breeds 12 polymorphisms (SNP) were identified within in the 5′-UTR (2/12), introns (5/12), and 3′-UTR (5/12) of SOX9 (Table 1). In addition an 18bp indel (Δ18) abolishing an interval between +247bp and +266bp downstream the TSS in four of the analysed pig breeds, i.e. German Large White, Laiwu Black, Bamei, and Erhualian, was identified (Table 2). As mentioned above we previously studied Δ18 in 545 animals of the commercial breeds in an attempt to identify an association with inguinal and scrotal hernia. However, no significant association could be detected using transmission disequilibrium test (p = 0.075) [52, 53]. All SNPs including Δ18 have been already deposited either with dbSNP or described previously as mentioned in the introduction [28].

Table 1. Polymorphisms detected in the porcine SOX9 gene.

| Position(a) | location  | dbSNP       |
|-------------|-----------|-------------|
| g.66309C>T  | 5′-UTR    | rs81219813  |
| g.66392G>T  | 5′-UTR    |             |
| g.66420_66437del | 5′-UTR |             |
| g.67588G>A  | intron 1  |             |
| g.67832T>A  | intron 1  | rs196959349 |
| g.68580C>G  | intron 2  | rs81219816  |
| g.68654G>T  | intron 2  | rs81219817  |
| g.68761C>T  | intron 2  | rs81219818  |
| g.70597T>G  | 3′-UTR    | rs327718481 |
| g.71244T>C  | 3′-UTR    | rs324747068 |
| g.71842C>T  | 3′-UTR    | rs322934388 |
| g.71902T>G  | 3′-UTR    | rs320099199 |
| g.71935A>G  | 3′-UTR    | rs337315481 |

(a) Positions according to GenBank accession number AC157866.2; variants according to HGVS http://www.hgvs.org/mutnomen/ (last accessed on May 29th, 2015)

doi:10.1371/journal.pone.0139583.t001
DNA sequence comparison between pig, cattle, buffalo, roe deer, and horse, demonstrates that the Δ18 region is highly conserved (Fig 1) and harbours three potential transcription factor binding sites with varying degrees of similarity to the respective consensus motives (Table 3). Due to the highly conserved sites and potential transcription factor binding sites, we hypothesized that this region might be important for SOX9 expression. To test CREB binding to the 18bp region in vivo, HEK293T cells were transfected with the CREB-expression plasmid (pcDNA4A-HA-CREB) and nuclear and cytoplasmic extracts were incubated with oligonucleotides harbouring the 18bp region (WT) and a CRE consensus sequence (CRE). As control an oligonucleotide homologous to the 18bp region with a mutated CRE half-site (MUT) was used. As shown in Fig 2ACREB binds to the WT and CRE oligonucleotides, whereas no binding was detected to the mutated control oligonucleotide. Specificity of CREB binding was analysed with competition and immunodepletion EMSAs. Nuclear and cytoplasmic extracts were prepared from ESK-4 cells and incubated with the labelled wild type DNA fragment (Fig 2B). As shown in Fig 2B, a specific band (BP) was detectable and diminished with increasing concentrations of cold unlabelled double strand homologous competitor. To exclude other factors which could bind to this region, we used antibodies directed against another potential transcription factor listed in Table 3, i.e. E2F, pre-incubated the cell extracts with the antibodies and then performed an immunodepletion EMSA as described elsewhere [54]. With the interference analysis we expected that due to binding of the antibody less transcription factor would be available to bind the labelled oligonucleotide and therefore would result in a reduced band intensity. Two antibodies were used, directed against CREB and E2F4. Fig 2C shows that when a polyclonal CREB antibody (anti-phospho-CREB) was added to the binding reaction the intensity of the protein-DNA complex was significantly reduced (lane 2) whereas an anti-E2F4 antibody did not influence the band intensity (lane 3). No binding was detected with the Δ18 fragment (lane 4). The band intensities were densitometrically quantified and differed significantly between lane 2 and 3 (Fig 2C lower panel).

To analyse whether CREB binding and the Δ18 variant in the context of the 5′-UTR have regulatory effects on SOX9 expression the wild type and the Δ18 5′-UTR were cloned into the pGL4.20 vector and used for transfection of HEK293T cells with or without a CREB expression plasmid. As shown in Fig 3 (left panel, -CREB) the wild type 5′-UTR resulted in a significantly higher activity than the Δ18 fragment in the absence of CREB. When CREB was co-expressed

| Breed                          | n  | wt/wt | Δ18/wt | Δ18/Δ18 |
|-------------------------------|----|-------|--------|---------|
| Different commercial breeds²) | 665| 388   | 235    | 42      |
| Erhualian                     | 59 | 58    | 1      | 0       |
| Laiwu Black                   | 37 | 25    | 7      | 5       |
| Bamei                         | 35 | 23    | 7      | 5       |
| Wuzhishan                     | 29 | 29    | 0      | 0       |
| Hang                          | 43 | 43    | 0      | 0       |
| Jianhexiang White             | 28 | 28    | 0      | 0       |
| Tibetan (Milinag)             | 42 | 42    | 0      | 0       |

Table 2. Genotype distribution of the 18-bp variant in different commercial breeds, seven Chinese domestic breeds and three Western commercial breeds.

- a) Commercial breeds included Pietrain, German Landrace, Large White, Red Duroc;
- b) pigs harbouring the wild type allele (homozygous);
- c) Δ18: pigs harbouring the 18-bp deletion allele (homozygous).

doi:10.1371/journal.pone.0139583.t002
Fig 1. Organization and location of the proximal promoter of the porcine SOX9 gene. The 18bp sequence and the CRE half-site are indicated. The sequence alignment in the lower panel shows conserved regions between pig, cattle, buffalo, roe deer, and horse. (B) Sequence alignment of the wild type and 18bp indel (Δ18) region.

doi:10.1371/journal.pone.0139583.g001
(Fig 3 right panel, +CREB) the activity of the wild type 5′-UTR was reduced to the level of the Δ18 fragment and both fragments resulted in a significant lower expression than the CREB induced expression of the control vector.

Effects of the variable SOX9 5′-UTR on transcription and translation in different cell types

Three expression vectors were constructed, i.e. psiCHECK-2-TTG, -WT-TTG and Δ18-TTG and transfected into HEK293T, PK–15, and ATDC5 cells. As shown in Fig 4A the Renilla luciferase start codon was mutated to TTG (psiCHECK-2-TTG) to abolish translation from this site. As the 5′-UTR of porcine SOX9 with a length of 394 bp does not contain multiple open reading frames or other upstream AUGs except the natural start codon, we cloned the two different variants of the SOX9 5′-UTR into the NheI cloning site of the psiCHECK-2-TTG vector to generate psiCHECK-2-WT-TTG and psiCHECK-2-Δ18-TTG. In these constructs translation will be initiated from the original SOX9 start codon -12 bp upstream with the addition of 4 amino acids at the N-terminus of the Renilla luciferase. Although the N-terminus of the luciferase is important for the stability of the enzyme [55], it has been shown that the addition of amino acids, e.g. Myc epitope or His-tag, does not influence the activity [56, 57].

In all three transfected cell types the luciferase activity was almost reduced to background levels compared to the intact expression vector demonstrating the effect of the mutated Renilla start codon (Fig 4B). Renilla luciferase activity of psiCHECK-2-TTG was then used to assess the effect of the 5′-UTR on translation. Both constructs harbouring the wild type (WT) or Δ18 were used for transfection of HEK293T, PK–15, and ATDC5 cells. In all three cell types, the Δ18 showed a significant lower luciferase activity compared to the wild type 5′-UTR construct. In addition, Renilla transcripts were analysed by qRT-PCR. As shown in Fig 5 the wild type 5′-UTR resulted in a 3-fold higher RNA concentration compared to Δ18.

Discussion

In the present study we characterized the effects of an 18bp deletion within the 5′-UTR of porcine SOX9 on transcription and translation. A sequence comparison of the 18bp region between pig and other species showed several conserved nucleotide positions including a CRE half-site. To determine whether the CRE half-site was recognized by CREB, we performed affinity purification, competition and immunodepletion mobility shift assays and were able to show binding. CREB did functionally bind to the CRE half-site (CGTCA/TGACG), albeit with
**Fig 2. CREB binds the CRE half-site in the 18bp region.** (A) Biotinylated oligonucleotides WT, MUT, and CRE were incubated with cell extracts of HEK293T cells transfected with a CREB expression plasmid. Bound protein was purified using Streptavidin MagneSphere Paramagnetic particles and a MagneSphere Magnetic Separation Stand (Promega), separated on an SDS/PAGE and Western blotted. Western blots were incubated with an HA-CREB antibody. Intensities of bands were densitometrically measured using ImageJ software (http://imagej.nih.gov/ij/index.html). (B) A constant amount of nuclear and cytoplasmic proteins of ESK-4 cells and labelled probe (WT) were incubated with increasing amounts of homologous unlabelled competitor. DsFP, double stranded free probe; ssFP: single stranded free probe; BP, bound protein-DNA complexes. (C) Immunodepletion assays were performed using anti-phospho-CREB and anti-E2F4 antibodies. Density of the resulting bands was determined using ImageJ software.

doi:10.1371/journal.pone.0139583.g002
reduced binding affinity compared to the full palindrome (TGACGTCA) [58]. CREB seems to be an important transcription factor in the regulation of bone development by activating Sox9 in mouse. Overexpression of Creb enhanced the action of Sox9 in the PKA pathway in BMP-2-induced osteochondrogenic differentiation [59]. Potent dominant negative Creb transgenic mice showed short-limbed dwarfism along with a defect in chondrocyte proliferation and a delay in chondrocyte hypertrophy in endochondral bone formation [60]. Other sites could also interfere with the expression, for example, an additional conserved CRE half-site at position -147 in the human SOX9 promoter [37]. However, CREB binding in the porcine SOX9 gene 5′-UTR seems to down-regulate expression as shown in Fig 3. Interestingly, CREB co-expression clearly up-regulated the promoterless control vector pGL4.20. Although it has been reported that pRL-TK used as normalizer is up-regulated by different transcription factors, e.g. GATA transcription factors [49], 12S E1A [61], SP1 [51], Nurr1 [50], and dexamethasone [62], there are no data available that CREB influences pRL-TK expression per se. However, as it is known that the TK promoter is influenced by CREB binding [63], we also expected that pRL-TK will be up-regulated and therefore took this into account when normalizing the expression levels. Hence, the increased pGL4.20 expression in fact seems to be due to a CREB dependent induction. Although this was an unexpected and so far unknown effect of CREB on pGL4.20 expression, promotorless luciferase reporter vectors pGL3 and pGL4 have been reported to be at least responsive to steroid hormones [64].
Inhibition of expression by CREB has been shown for other genes, e.g., MuSK, AP-2α, and PPAR-γ [65–67]. Truncations or mutations in UTRs often defeat fine regulation, cause impaired protein synthesis and finally associate with various diseases or disease susceptibility in humans [68]. Two studies showed that microRNA–145 directly repressed SOX9 expression
by binding a unique site in the 3'-UTR resulting in a profound change in the human chondrocyte phenotype [69, 70]. It is also known that a stable secondary structure, multiple ORFs, and uAUGs in the 5'-UTR largely determine translation efficiency [71]. However, the 5'-UTR of the porcine SOX9 has only one ORF and hence the downregulation of SOX9 expression seems to be due only to either CREB binding or the presence of Δ18. CREB binding resulted in a reduction of SOX9 transcription as shown in Fig 3, whereas Δ18 could have an effect on either RNA stability or RNA degradation. A comparative in silico analysis of the secondary structure formation of the wild type and Δ18 5'-UTR showed that wild type RNA had a lower ΔG (-170.77) than Δ18 (-161.78) (data not shown).

Although regulation of SOX9 expression is complex and the role of a single site must be interpreted with caution, our results are in agreement with the findings that the wild type 5'-UTR was identified more often in 38,XX sex-reversal pigs and SOX9 is over-expressed in XX intersex gonads [23, 28]. As shown in Figs 4 and 5 the presence of the 18bp region resulted in a significant increase of promoter activity compared to Δ18. Only, when CREB was over-expressed and bound to its binding site a reduction of expression was observed (Fig 3). From these findings different scenarios for the development of a 38,XX sex-reversal phenotype can be hypothesized. In the presence of the wild type genotype a sex reversal phenotype could result from a low CREB expression and a more stable SOX9 RNA secondary structure leading to high SOX9 concentrations and consequently to a male phenotype. On the other hand, if CREB expression is high enough to down-regulate SOX9 expression, no sex reversal will be induced. In the presence of the Δ18 genotype, where SOX9 expression was low independent of CREB and the RNA secondary structure was less stable, a normal sex development would be expected for 38,XX individuals.

Hence, in further experiments it will be interesting to analyse the expression of CREB in controls and 38,XX sex-reversal gonads.

Fig 5. Determination of wild type and Δ18 5'-UTR transcription regulation. RT-qPCR was performed for quantifying Renilla mRNA contents. The content of Renilla was normalized to β-actin as internal control and the relative luciferase mRNA for psiCHECK-2-TTG was fixed as 1.

doi:10.1371/journal.pone.0139583.g005
Conclusion
We have shown that an 18bp deletion located +248 bp downstream the TSS in the 5′-UTR of the porcine SOX9 gene harbours a functional CRE half-site. The deletion segregates in several pig breeds. The 18bp region is essential for transcription and translation efficiency of porcine SOX9 and interacts with CREB. CREB binding to the wild type or presence of the 18bp deletion results in a significant reduction of SOX9 transcription and translation.

Acknowledgments
The authors are thankful to Ch. Knorr for support and encouragement at the beginning of the project. S. Pach is thanked for expert technical assistance.

Author Contributions
Conceived and designed the experiments: BB YD YX ND. Performed the experiments: YD YX ND. Analyzed the data: BB YD YX ND ES. Contributed reagents/materials/analysis tools: BB LH. Wrote the paper: BB YD ES.

References
1. Jo A, Denduluri S, Zhang B, Wang Z, Yin L, Yan Z, et al. The versatile functions of Sox9 in development, stem cells, and human diseases. Genes Dis. 2014; 1(2):149–61. doi: 10.1016/j.gendis.2014.09.004 PMID: 25685828; PubMed Central PMCID: PMC4326072.
2. Wuelling M, Vortkamp A. Chondrocyte proliferation and differentiation. Endocrine development. 2011; 21:1–11. Epub 2011/08/26. doi: 10.1159/000328081 PMID: 21865749.
3. Jakob S, Lovell-Badge R. Sex determination and the control of Sox9 expression in mammals. FEBS J. 2011; 278(7):1002–9. doi: 10.1111/j.1742-4658.2011.08029.x PMID: 21281448.
4. Passeron T, Valencia JC, Bertolotto C, Hoashi T, Le Pape E, Takahashi K, et al. SOX9 is a key player in ultraviolet B-induced melanocyte differentiation and pigmentation. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104(35):13984–9. doi: 10.1073/pnas.0705117104 PMID: 17702866; PubMed Central PMCID: PMC2195578.
5. Raspopovic J, Marcon L, Russo L, Sharpe J. Modeling digits. Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. Science. 2014; 345(6196):566–70. doi: 10.1126/science.1252960 PMID: 25082703.
6. Harris ML, Baxter LL, Loftus SK, Pavan WJ. Sox proteins in melanocyte development and melanoma. Pigment Cell Melanoma Res. 2010; 23(4):496–513. doi: 10.1111/j.1755-148X.2010.00711.x PMID: 20444197; PubMed Central PMCID: PMC2906668.
7. Leung VY, Gao B, Leung KK, Melhado IG, Wynn SL, Au TY, et al. SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct concomitant transactivation and repression. PLoS genetics. 2011; 7(11):e1002356. doi: 10.1371/journal.pgen.1002356 PMID: 22072985; PubMed Central PMCID: PMC3207907.
8. Moniot B, Biau S, Faure S, Nielsen CM, Berta P, Roberts DJ, et al. SOX9 specifies the pyloric sphincter epithelium through mesenchymal-epithelial signals. Development (Cambridge, England). 2004; 131 (15):3795–804. doi: 10.1242/dev.01259 PMID: 15240557; PubMed Central PMCID: PMC2442161.
9. Purba TS, Haslam IS, Shahmalak A, Bhogal RK, Paus R. Mapping the expression of epithelial hair follicle stem cell-related transcription factors LH2X and SOX9 in the epidermal hair follicle. Exp Dermatol. 2015; 24(6):462–7. doi: 10.1111/exd.12700 PMID: 25808706.
10. Daigle M, Rouaud P, Martin LJ. Expressions of Sox9, Sox5, and Sox13 transcription factors in mice testis during postnatal development. Mol Cell Biochem. 2015. doi: 10.1007/s11010-015-2470-7 PMID: 26046173.
11. Frojdman K, Harley VR, Pelliniemi LJ. Sox9 protein in rat sertoli cells is age and stage dependent. Histochim Cell Biol. 2000; 113(1):31–6. PMID: 10664067.
12. Faucette AN, Maher VA, Gutierrez MA, Jucker JM, Yates DC, Welsh TH Jr., et al. Temporal changes in histomorphology and gene expression in goat testes during postnatal development. Journal of animal science. 2014; 92(10):4440–8. doi: 10.2527/jas.2014-7903 PMID: 25085396.
13. Akiyama H. Control of chondrogenesis by the transcription factor Sox9. Mod Rheumatol. 2008; 18(3):213–9. Epub 2008/03/21. doi: 10.1007/s10165-008-0046-x PMID: 18351289.
14. Stuhlmiller TJ, Garcia-Castro ML. Current perspectives of the signaling pathways directing neural crest induction. Cell Mol Life Sci. 2012; Epub 2012/05/02. doi: 10.1007/s00018-012-0991-8 PMID: 22547091.

15. Zalzali H, Naudin C, Quittat-Prevostel C, Yaghi C, Poulat F, et al. CEACAM1, a SOX9 direct transcriptional target identified in the colon epithelium. Oncogene. 2008; 27(56):7131–8. Epub 2008/09/17. doi: 10.1038/onc.2008.331 PMID: 18794798.

16. Pritchett J, Athwal V, Roberts N, Hanley NA, Hanley KP. Understanding the role of SOX9 in acquired diseases: lessons from development. Trends Mol Med. 2011; 17(3):166–74. doi: 10.1016/j.trendsmm.2010.12.001 PMID: 21237710.

17. Lee YH, Saint-Jeannet JP. Sox9 function in craniofacial development and disease. Genesis. 2011; 49(4):200–8. doi: 10.1002/dvg.20717 PMID: 21309066; PubMed Central PMCID: PMC3079054.

18. Gordon CT, Tan TY, Benko S, Fitzpatrick D, Lyonnet S, Farlie PG. Long-range regulation at the SOX9 locus in development and disease. J Med Genet. 2009; 46(10):649–56. doi: 10.1136/jmg.2009.068361 PMID: 1947998.

19. Brennan CB, Buehler T, Lesher JL Jr. Cooks syndrome: a case report and brief review. Pediatr Dermatol. 2013; 30(4):e52–3. doi: 10.1111/j.1525-1470.2011.01668.x PMID: 22329539.

20. Ostrer H. Disorders of sex development (DSDs): an update. J Clin Endocrinol Metab. 2014; 99(5):1503–9. doi: 10.1210/jc.2013-3690 PMID: 24758178.

21. Sun M, Li N, Dong W, Chen Z, Liu Q, Xu Y, et al. Copy-number mutations on chromosome 17q24.2-q24.3 in congenital generalized hypertrichosis terminalis with or without gingival hyperplasia. Am J Hum Genet. 2009; 84(6):807–13. doi: 10.1016/j.ajhg.2009.04.018 PMID: 19463983; PubMed Central PMCID: PMC2694973.

22. Kropatsch R, Dekomien G, Akkad DA, Gerding WM, Petrasch-Parwez E, Young ND, et al. SOX9 duplication linked to intersex in deer. PloS One. 2013; 8(9):e73734. doi: 10.1371/journal.pone.0073734 PMID: 24040047; PubMed Central PMCID: PMC3765313.

23. Pailhoux E, Parma P, Sundstrom J, Figier B, Servel N, Kuopio T, et al. Genetic analysis of 38XX males with genital ambiguities and true hermaphrodites in pigs. Animal Genetics. 1999; 30(4):150–3. doi: 10.1111/j.1525-1470.1999.01668.x PMID: 22329539.

24. Pailhoux E, Popescu PC, Parma P, Boscher J, Legault C, Molteni L, et al. Genetic analysis of 38XX males with genital ambiguities and true hermaphrodites in pigs. Animal Genetics. 2013; 44(8):781–90. doi: 10.1111/j.1365-2052.2013.02763.x PMID: 24422202; PubMed Central PMCID: PMC3819277.

25. Kothapalli K, Kirkness E, Pajar S, Van Wormer R, Meyers-Wallen VN. Exclusion of candidate genes for canine SRY-negative XX sex reversal. J Hered. 2005; 96(7):759–64. doi: 10.1093/jhered/esi129 PMID: 16267164.

26. Ross E, Radi O, De Lorenzi L, Vetro A, Groppetti D, Bigliardi E, et al. Sox9 duplications are a relevant cause of Sry-negative XX sex reversal dogs. PloS One. 2014; 9(7):e101244. doi: 10.1371/journal.pone.0101244 PMID: 25010117; PubMed Central PMCID: PMC4091935.

27. Kimura R, Murata C, Kuroki Y, Kuroiwa A. Mutations in the testis-specific enhancer of SOX9 in the SRY independent sex-determining mechanism in the genus Tokudaia. PloS One. 2014; 9(9):e108779. doi: 10.1371/journal.pone.0108779 PMID: 25265165; PubMed Central PMCID: PMCPMC4181316.

28. Mead TJ, Wang Q, Bhattaram P, Dy P, Afeleik S, Jensen J, et al. A far-upstream (-70 kb) enhancer mediates Sox9 auto-regulation in somatic tissues during development and adult regeneration. Nucleic acids research. 2013; 41(8):4459–69. doi: 10.1093/nar/gkt4222; PubMed Central PMCID: PMCPMC3632127.

29. Mertin S, McDowell SG, Harley VR. The DNA-binding specificity of SOX9 and other SOX proteins. Nucleic acids research. 1999; 27(5):1359–64. PMID: 9973626; PubMed Central PMCID: PMC148324.
34. Kanai Y, Koopman P. Structural and functional characterization of the mouse Sox9 promoter: implications for campomelic dysplasia. Hum Mol Genet. 1999; 8(4):691–6. Epub 1999/03/11. PMID: 10072439.

35. Colter DC, Piera-Velazquez S, Hawkins DF, Whitecavage MK, Jimenez SA, Stokes DG. Regulation of the human Sox9 promoter by the CCAAT-binding factor. Matrix Biol. 2005; 24(3):185–97. Epub 2005/05/24. doi: 10.1016/j.matbio.2005.04.001 PMID: 15908194.

36. Piera-Velazquez S, Hawkins DF, Whitecavage MK, Colter DC, Stokes DG, Jimenez SA. Regulation of the human SOX9 promoter by Sp1 and CREB. Exp Cell Res. 2007; 313(6):1069–79. Epub 2007/02/10. doi: 10.1016/j.yexcr.2007.01.001 PMID: 17289023; PubMed Central PMCID: PMC2118054.

37. Piera-Velazquez S, Hawkins DF, Whitecavage MK, Colter DC, Stokes DG, Jimenez SA. Regulation of the human SOX9 promoter by Sp1 and CREB. Experimental cell research. 2007; 313(6):1069–79. Epub 2007/02/10. doi: 10.1016/j.yexcr.2007.01.001 PMID: 17289023; PubMed Central PMCID: PMC2118054.

38. Colter DC, Piera-Velazquez S, Hawkins DF, Whitecavage MK, Jimenez SA, Stokes DG. Regulation of the human SOX9 promoter by the CCAAT-binding factor. Matrix biology: journal of the International Society for Matrix Biology. 2005; 24(3):185–97. Epub 2005/05/24. doi: 10.1016/j.matbio.2005.04.001 PMID: 15908194.

39. Montminy MR, Bilezikjian LM. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature. 1987; 328(6126):175–8. doi: 10.1038/328175a0 PMID: 2885756.

40. Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P. A male-specific role for SOX9 in vertebrate sex determination. Development (Cambridge, England). 1996; 122(9):2813–22. PMID: 8787755.

41. Murakami S, Kan M, McKeehan WL, de Crombrugghe B. Up-regulation of the chondrogenic Sox9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97(3):1113–8. PMID: 10655493; PubMed Central PMCID: PMC15539.

42. Caron MM, Emans PJ, Cremers A, Coolsen MM, van Rhijn LW, et al. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP–2 but suppressed by BMP–7. Osteoarthritis Cartilage. 2013; 21(4):604–13. doi: 10.1016/j.joca.2013.01.009 PMID: 23936668.

43. Chen X, Shi X, Gan F, Huang D, Huang K. Glutamine starvation enhances PCV2 replication via the phosphorylation of p38 MAPK, as promoted by reducing glutathione levels. Vet Res. 2015; 46:32. doi: 10.1186/s13566-015-0168-1 PMID: 25879878; PubMed Central PMCID: PMCPMC463047.

44. Guo H, Hao R, Wei Y, Sun D, Sun S, Zhang Z. Optimization of electrotransfection conditions of mammalian cells with different biological features. J Memb Biol. 2012; 245(12):789–95. doi: 10.1007/s00232-012-9480-0 PMID: 22836669.

45. Roman AC, Benitez DA, Carvajal-Gonzalez JM, Fernandez-Salgueiro PM. Genome-wide B1 retrotransposon binds the transcription factors dioxin receptor and Slug and regulates gene expression in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105(5):1632–7. doi: 10.1073/pnas.0708366105 PMID: 18223496; PubMed Central PMCID: PMCPMC223496.

46. Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. Nat Protoc. 2007; 2(8):1849–57. doi: 10.1038/nprot.2007.249 PMID: 17703195; PubMed Central PMCID: PMC2757439.

47. Arora A, Dutkiewicz M, Scaria V, Hariharan M, Maiti S, Kurreck J. Inhibition of translation in living eukaryotic cells by an RNA G-quadruplex motif. RNA (New York, NY). 2008; 14(7):1290–7. doi: 10.1261/rna.1001708 PubMed Central PMCID: PMCPMC2441988.

48. Kubokawa I, Takeshima Y, Ota M, Enomoto M, Okizuka Y, Mori T, et al. Molecular characterization of the 5′-UTR of retinylidrophin reveals a cryptic intron that regulates translational activity. Molecular vision. 2010; 16:2590–7. Epub 2010/12/15. PubMed Central PMCID: PMCPMC300234.

49. Ho CK, Strauss JF 3rd. Activation of the control reporter plasmids pRL-TK and pRL-SV40 by multiple GATA transcription factors can lead to aberrant normalization of transfection efficiency. BMC Biotechnol. 2004; 4:10. doi: 10.1186/1472-6750-4-10 PMID: 15119957; PubMed Central PMCID: PMCPMC416485.

50. Matuszyk J, Ziolo E, Cebrat M, Kochel I, Strzadala L. Nurr1 affects pRL-TK but not phRG-B internal control plasmid in genetic reporter system. Biochem Biophys Res Commun. 2002; 294(5):1036–9. doi: 10.1016/S0006-291X(02)00601-0 PMID: 12074581.

51. Osborne SA, Tonissen KF. pRL-TK induction can cause misinterpretation of gene promoter activity. Biotechniques. 2002; 33(6):1240–2. PMID: 12503307.

52. Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). Am J Hum Genet. 1993; 52(3):506–16. PMID: 8447318; PubMed Central PMCID: PMCPMC1682161.
53. Xing Y. Dissection of genetic variants affecting boar sperm quality and porcine inguinal/scrotal hernia. Göttingen: Cuvillier Verlag Göttingen; 2010. 110 p.

54. Dyer RB, Herzog NK. Immunodepletion EMSA: a novel method to identify proteins in a protein-DNA complex. Nucleic acids research. 1995; 23(16):3345–6. PMID: 7667113; PubMed Central PMCID: PMC307198.

55. Sung D, Kang H. The N-terminal amino acid sequences of the firefly luciferase are important for the stability of the enzyme. Photochem Photobiol. 1998; 68(5):749–53. Epub 1998/11/24. PMID: 9825705.

56. Loening AM, Fenn TD, Wu AM, Gambhir SS. Consensus guided mutagenesis of Renilla luciferase yields enhanced stability and light output. Protein Eng Des Sel. 2006; 19(6):391–400. Epub 2006/07/22. doi: 10.1093/protein/gzl023 PMID: 16857694.

57. Sung D, Kang H. The N-terminal amino acid sequences of the firefly luciferase are important for the stability of the enzyme. Photochem Photobiol. 1998; 68(5):749–53. Epub 1998/11/24. PMID: 9825705.

58. Long F, Schipani E, Asahara H, Kronenberg H, Montminy M. CREB family of activators is required for endochondral bone development. Development (Cambridge, England). 2001; 128(4):541–50. Epub 2001/02/15.

59. Zhao L, Li G, Zhou GQ. SOX9 directly binds CREB as a novel synergism with the PKA pathway in BMP-2-induced osteochondrogenic differentiation. Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research. 2009; 24(5):826–36. Epub 2008/12/31. doi: 10.1359/jbmr.081236 PMID: 1908894.

60. Thavathiru E, Das GM. Activation of pRL-TK by 12S E1A oncoprotein: drawbacks of using an internal reference reporter in transcription assays. Biotechniques. 2001; 31(3):528–30. PMID: 11570496.

61. Ibrahim NM, Marinovic AC, Price SR, Young LG, Frohlich O. Pitfall of an internal control plasmid: response of Renilla luciferase (pRL-TK) plasmid to dihydrotestosterone and dexamethasone. Biotechniques. 2000; 39(2):203–7. PMID: 11056808.

62. Herzig S, Hedrick S, Morantte I, Koo SH, Galimi F, Montminy M. CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. Nature. 2003; 426(6963):190–3. doi:10.1038/nature02110 PMID: 14614508.

63. Kim CH, Xiong WC, Mei L. Inhibition of MuSK expression by CREB interacting with a CRE-like element and MyoD. Molecular and cellular biology. 2005; 25(13):5329–38. doi: 10.1128/MCB.25.13.5329-5338.2005 PMID: 15964791; PubMed Central PMCID: PMC1156998.

64. Melnikova VO, Dobroff AS, Zigler M, Villares GJ, Braeuer RR, Wang H, et al. CREB inhibits AP-2alpha expression to regulate the malignant phenotype of melanoma. PloS one. 2010; 5(8):e12452. doi: 10.1371/journal.pone.0012452 Epub 2010/08/10.

65. Chatterjee S, Pal JK. Role of 5'- and 3'-untranslated regions of mRNAs in human diseases. Biology of the cell / under the auspices of the European Cell Biology Organization. 2009; 101(5):251–62. Epub 2009/03/12. doi: 10.1042/bc20080104

66. Yang B, Guo H, Zhang Y, Chen L, Ying D, Dong S. MicroRNA−145 regulates chondrogenic differentiation of mesenchymal stem cells by targeting Sox9. PloS one. 2011; 6(7):e21679. Epub 2011/07/30. doi: 10.1371/journal.pone.0021679 PubMed Central PMCID: PMC3140487.

67. Martinez-Sanchez A, Dudek KA, Murphy CL. Regulation of human chondrocyte function through direct inhibition of cartilage master regulator Sox9 by microRNA−145 (miRNA−145). The Journal of biological chemistry. 2012; 287(2):916–24. Epub 2011/11/22. doi: 10.1074/jbc.M111.302430 PubMed Central PMCID: PMC3256897.

68. Meng Z, Wang J, Wang X, Wang J, Yuan Y, Zhang G, et al. Identification of novel miRNAs and their potential targets in human hepatocellular carcinoma. PloS one. 2013; 8(9):e73852. doi: 10.1371/journal.pone.0073852

69. Quan H, Li L, Zhang S, Li J, Guo H, Zhang Q, et al. MiR-145 interacts with SOX9 and regulates chondrogenesis in human mesenchymal stem cells. PloS one. 2014; 9(11):e111542. doi: 10.1371/journal.pone.0111542

70. Kozak M. Structural features in eukaryotic mRNAs that modulate the initiation of translation. The Journal of Biological Chemistry. 1991; 266(30):19867–70. Epub 1991/10/25. PMID: 1939050