Constitutive L-Sox5 overexpression delays differentiation of ATDC5 cells into chondrocytes and correlates with reduced expression of differentiation markers

Dongmei Sun, **Vishwa Deepak**, **Ping Mu**, **Haiying Jiang**, **Xiuming Shi**, **Zhongbo Liu**, **Xianlu Zeng**, **Wenguang Liu**

Abstract L-Sox5 is a member of sex-determining region Y-type high mobility group box (SOX) family of transcription factors. We assessed the effects of retroviral overexpression of L-Sox5 on chondrocyte differentiation using the clonal murine cell line ATDC5. We observed a temporal-restricted expression pattern of L-Sox5 in insulin-induced ATDC5 cells differentiating toward chondrocyte lineage. The protein expression levels of L-Sox5 showed a drastic decrease in contrast to unaltered mRNA levels during differentiation. L-Sox5 delayed the differentiation of ATDC5 cells as evidenced by Alcian blue staining for proteoglycan synthesis. The mRNA levels of chondrocyte and hypertrophic/osteoarthritic markers were markedly decreased or delayed in L-Sox5 overexpressing cells. L-Sox5 abrogated the promoter activity of Runx2. These results suggest that L-Sox5 protein expression may diminish along with the progress of chondrogenic differentiation. L-Sox5 may act as a negative regulator if expressed aberrantly at least in part by regulating the critical fate of chondrogenesis.

Keywords L-Sox5 · Chondrogenesis · Runx2 · Hypertrophy · Asporin · Osteoarthritis

D. Sun · P. Mu · H. Jiang · X. Shi · Z. Liu · X. Zeng · W. Liu
Key Laboratory of Molecular Epigenetics of Ministry of Education of China, Northeast Normal University, Renmin Street 5268, Changchun 130024, Jilin, China
e-mail: lwgqj@hotmail.com; liuwg788@nenu.edu.cn

V. Deepak
Department of Physiology, Faculty of Health Sciences, School of Medicine, University of Pretoria, Private Bag X323, Arcadia, Pretoria 0007, South Africa
e-mail: vishwa.deepak@up.ac.za

W. Liu
Institute of Genetics and Cell Biology, Northeast Normal University, Changchun, Jilin, China

Introduction

Endochondral ossification is a complex and multistep process involving gradual replacement of embryonic cartilaginous model by bone. Mesenchymal precursor cells condense to form an anlage for the endochondral skeleton. The condensed cells initiate as proliferating chondrocytes, become prehypertrophic chondrocytes and end as hypertrophic chondrocytes [1]. Proliferating chondrocytes secrete extracellular matrix (ECM) proteins such as type II Collagen and Aggrecan; chondrocytes then exit the cell cycle, turn prehypertrophic, and synthesize type X collagen [1]. As hypertrophic chondrocytes become apoptotic, blood vessels invade the cartilaginous template that is eventually replaced by bone.

The sex-determining region Y-type high mobility group box (Sox) family transcription factors, L-Sox5, Sox6, and Sox9 (Sox trio), have been reported to tightly regulate chondrocyte differentiation and cartilage development [2]. Sox9 is the master transcription factor for chondrocyte lineage commitment and differentiation. Campomelic dysplasia in humans occurs due to heterozygous mutations in the gene whereas its knock out in mice results in complete absence of chondrocyte and bone [3]. L-Sox5 (a longer isoform of Sox5) and Sox6 are the two other members of the Sox family and function as co-activators of Sox9 during chondrogenesis [4]. L-Sox5 and Sox6 have

essential but redundant roles in cartilage development [2]. Even though combination of L-Sox5, Sox6, and Sox9 have been shown to be sufficient to induce and maintain overt chondrogenesis in cultured cells, growing body of evidence suggests that they inhibit the terminal differentiation stage of chondrocytes [5]. Nevertheless, the precise mode of action of the Sox trio is not completely understood till date due to the fact that they are involved in successive multiple steps of the complex chondrocyte differentiation pathway. Since, the Sox genes are essential for cartilage development, understanding the role of L-Sox5 would provide novel insights for advancing cartilage biology.

ATDC5 is a mouse embryonal carcinoma-derived cell line that mimics the chondrocyte differentiation processes from chondroprogenitors to fully differentiated and hypertrophic chondrocytes in a sequential, temporally regulated manner [6]. These cells are less phenotypically diverse than primary cultures and therefore widely used as a model to study in vitro chondrogenesis [7]. Upon stimulation with insulin ATDC5 cells form nodule-like aggregates and express chondrogenic markers (type II Collagen and sulfated Glycosaminoglycans) and further differentiate to become hypertrophic and express type X collagen [7]. The sequential changes that occur during early and late chondrogenesis are critical for the fate of different chondrocytes. Since L-Sox5 is a major Sox family member, we assessed its role in chondrocyte differentiation. We demonstrate that overexpression of L-Sox5 in ATDC5 cells delays fully chondrogenic differentiation and correlates with reduced expression of chondrogenic and hypertrophic/osteoarthritic markers.

Materials and methods

Reagents and antibodies

Insulin (#I0516), human transferrin (#T3309), sodium selenite (#S5261), ascorbate 2-phosphate (#A8960), anti-FLAG anti-bodies (#F1804), blasticidine (#15205), puromycin (#P8833), and Alcian blue 8GX (#A3157) were all purchased from Sigma. G418 (#345810) was bought from Merck.

Cell culture

The mouse chondroprogenitor cell line ATDC5 was maintained in DMEM/F12 (1:1) (GIBCO) containing 5 % fetal bovine serum (FBS; Hyclone), 10 $\mu g\ m l^{-1}$ human transferrin, 30 nM sodium selenite, 37.5 $\mu g\ m l^{-1}$ ascorbate 2-phosphate, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin. For chondrocyte differentiation, cells (5×10^4/well) were seeded and cultured to reach 100 % confluency (this time point was regarded as day 0 for subsequent differentiation), then medium was supplemented with 10 $\mu g\ m l^{-1}$ insulin and cells were cultured for up to 14 days. PLAT-E packaging cell lines were main-tained in DMEM containing 10 % FBS, 1 $\mu g\ m l^{-1}$ puro-mycin, 10 $\mu g\ m l^{-1}$ blasticidine, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin in 6-well plates coated with rat tail type I Collagen (Upstate).

Plasmid construction

The plasmid vector pcDNA 5′UT-FLAG and cDNA clone for L-Sox5 in the same vector were kindly provided by Dr. Lefebvre [4]. For constructing the control pMXs retroviral vector, a blunt HindIII-XbaI fragment containing the FLAG tag from pcDNA 5′UT-FLAG was introduced into the blunt EcoRI ends of pMXs/neo. This empty construct was used to generate the control cell line in this study. To introduce the FLAG-tagged L-Sox5 into the pMXs retroviral vector, the blunt HindIII-XbaI fragment containing L-Sox5 was ligated to the blunt EcoRI ends of pMXs/neo. All the constructs were confirmed by restriction enzymatic mapping.

Generation of the ATDC5 cell lines overexpressing L-Sox5 and their mock control

To generate the ATDC5 stable cell lines for this study, we used a retroviral system. PLAT-E cells were transfected with the pMXs constructs using Fugene-6 transfection reagent (Roche) according to the manufacturer's instructions. After 72 h, medium was collected as virus-containing supernatant. Then the virus supernatant was filtered through a 0.45-μm filter (Millipore) and was mixed in 1000:1 with polybrene from a stock solution of 5 $m g\ m l^{-1}$ (Sigma). ATDC5 cells were seeded 1 day before infection and were transduced with the polybrene virus supernatant for 6 h followed by replacement with fresh medium. Twenty-four hours later, ATDC5 cells were subjected to selection under 400 $\mu g\ m l^{-1}$ G418. The stable cell lines were confirmed by Western blot using anti-FLAG antibodies.

RNA preparation and RT-PCR assay

Total RNA was extracted from cells with Trizol reagent (Invitrogen). Reverse transcription and PCR were performed using Improm-II reverse transcriptase (Promega) and Taq polymerase premix (Takara), as per manufacturer's instructions. The primers are shown in supplementary Table 1. PCR reaction was performed on a P×2 Thermal Cycler (Thermal Electron Corporation).

Alcian blue staining

To analyze differentiation, cells were washed with PBS twice, fixed with 10 % (v/v) formaldehyde in PBS at room

temperature for 20 min, and stained with 0.1 % Alcian blue 8GX in 0.1 N HCL overnight. The next day, cells were rinsed with distilled water and examined under a microscope.

Western blot

Cells were lysed in RIPA buffer supplemented with protease inhibitors. 30 μg of proteins was loaded into each lane and was separated by SDS-PAGE and subsequently transferred to a PVDF membrane. Membranes were blocked in 5 % non-fat milk to prevent non-specific binding followed by incu-bation with the respective primary antibodies. Results were visualized using the ECL Plus detection kit (GE Healthcare) according to the manufacturer's instructions.

Microarray analysis

A detailed description is provided in supplementary information.

Transient transfection and dual luciferase assay

The RUNX2 promoter and 5′-UTR region (−2859 to + 395), as illustrated in the sequence with genbank number AY090738, were amplified using high-fidelity polymerase and cloned into pREP4-Luc. 293T cells grown in 6-well plates were transfected by Fugene 6 transfection reagent with 0.5 μg of the RUNX2 promoter-luciferase construct along with 0.5 μg of the L-Sox5 expression construct or its empty vector [4]. Cells in each well were also cotrans-fected with 0.1 μg of pREP7-RLuc plasmids as an internal control. Relative luciferase activities were measured 40 h after transfection by the dual luciferase assay system (Promega) on a TD-20/20 luminometer (Turner Designs). For characterizing Sox6 and Sox9, the same methodology was employed as mentioned above for L-Sox5 assays. For L-Sox5 and Sox6 or L-Sox5 and Sox9 analysis, a total of 0.5 μg of respective plasmids (0.25 μg L-Sox5 and 0.25 μg Sox6 or Sox9) were cotransfected into the cells.

Results

Overexpression of L-Sox5 inhibited in vitro chondrogenesis

To delineate the function of L-Sox5 protein in chondrocyte differentiation, we established ATDC5 variant cell lines overexpressing L-Sox5 and their mock controls. The protein levels of L-Sox5 weakened after induction of chondrocyte differentiation (Fig. 1a); however, mRNA expression levels of L-Sox5 did remained unaltered throughout chondrocyte differentiation (Fig. 1b).

Furthermore, to examine the chondrogenic ability of the cell lines overexpressing L-Sox5 and their mock controls, Alcian blue staining for proteoglycan biosynthesis was performed. Alcian blue-positive cartilaginous nodules were detected for the control cells as earlier as day 7, and overt cartilaginous nodules were detected at day 14 (Fig. 1c). Intriguingly, even though L-Sox5 overexpression did not abolish the commitment to chondrocyte lineage, no Alcian blue-positive nodules were detected at day 7, and weaker Alcian blue-positive nodules were detected at day 14, for the L-Sox5 overexpressing cells (Fig. 1c).

L-Sox5 suppressed cartilage-specific markers gene expression, hypertrophic markers gene expression, and osteoarthritis-associated ECM protein's gene expression

RT-PCR assay was conducted to examine gene expression of the cartilage-specific matrix proteins during chondrocyte differentiation. As shown in Fig. 2a, L-Sox5

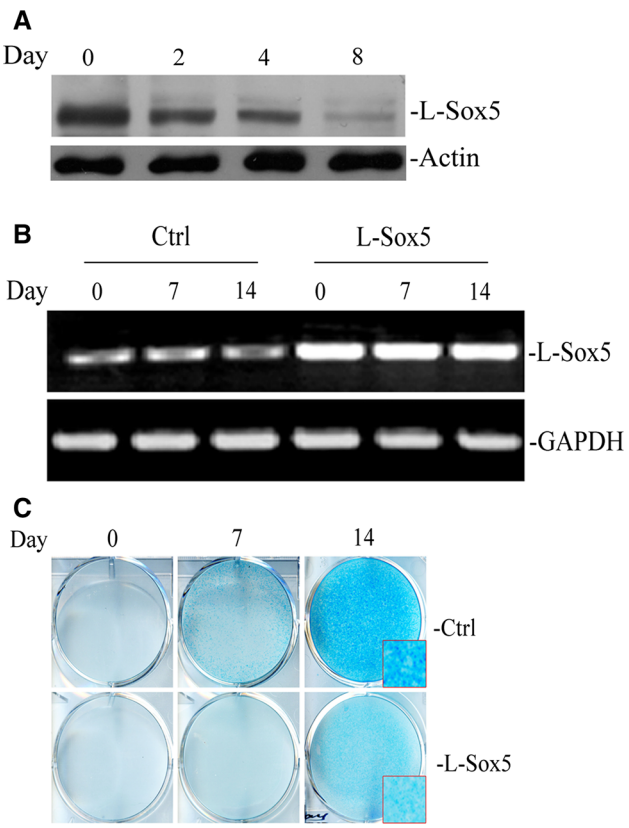

Fig. 1 Gene and protein expression levels of L-Sox5 during chondrogenesis in control and L-Sox5 overexpressing cell lines. **a** Change in the protein levels of L-Sox5 during chondrogenesis. Confluent cells at day 0 were subjected to chondrogenesis. **b** Gene expression of L-Sox5. Confluent cells at day 0 were subjected to chondrogenesis. **c** Chondrogenesis of the ATDC5 cell lines. Confluent cells at day 0 were subjected to chondrogenic differentiation. Differentiated cells that formed nodules were stained by Alcian blue

overexpression delayed or suppressed the gene expression of type II Collagen, Aggrecan, Comp, Lumican, Decorin, and Matrilin (chondrogenic markers). Furthermore, overexpression of L-Sox5 suppressed Runx2 and Asporin gene expression and delayed type X Collagen gene expression (Fig. 2b, c). We then performed microarray to investigate genes that were regulated by overexpression of L-Sox5 in ATDC5 cells (supplementary Table 2). Indeed, we also found that gene expression of Decorin and Lumican was identified to be decreased (top 10th and 15th, respectively, among the downregulated 395 genes shown in supplementary Table 2) by L-Sox5 overexpression. Concomitantly, microarray data confirmed that Asporin gene expression was dramatically decreased by L-Sox5 overexpression (top 8th among the downregulated 395 genes shown in supplementary Table 2).

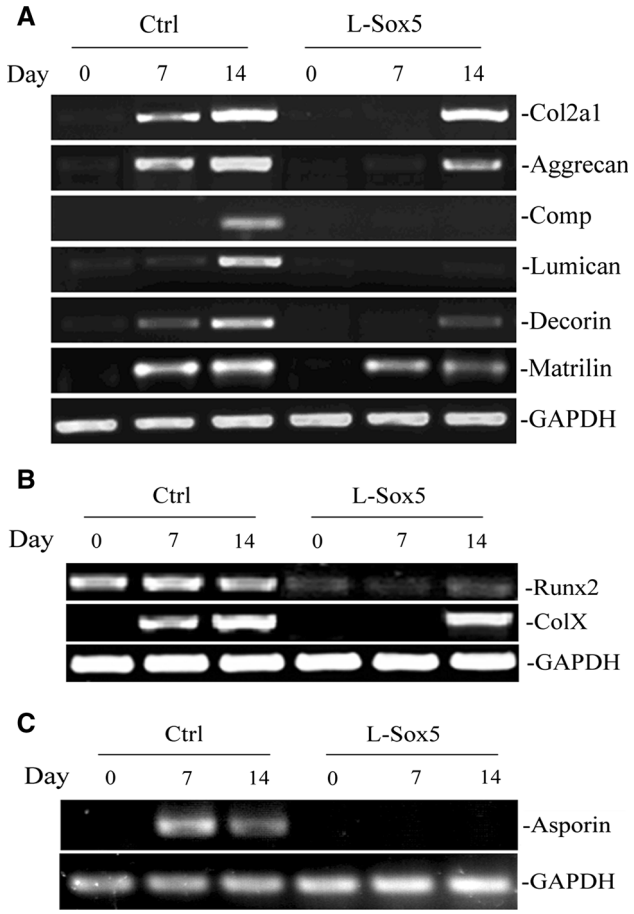

Fig. 2 Expression of cartilage-specific genes in control and L-Sox5 overexpressing cell lines during chondrogenesis. **a** Confluent cells at day 0 were subjected to chondrogenesis. Total RNA from each cell line was extracted and mRNA levels were determined by RT-PCR. **b**, **c** Gene expression of Runx2, ColX, and Asporin in the ATDC5 control and L-Sox5 overexpressing cell lines

L-Sox5 suppresses Runx2 promoter activity

To characterize the effects of L-Sox5 on chondrocyte hypertrophy, we performed dual luciferase assay. We found that L-Sox5 significantly inhibited the RUNX2 promoter activity (Fig. 3a). To exactly delineate if Sox6 could also have a suppressive effect on Runx2 promoter activity, Sox6 plasmid either alone or in combination with L-Sox5 was cotransfected and assessed for promoter activity. Sox6-mediated repression of Runx2 promoter activity was similar to Sox5 repression as well as cotransfection of these plasmids did not display a sup-pressive effect greater than the plasmids when transfected alone (Fig. 3b). To find out whether L-Sox5 activity is dependent or independent of Sox9, another reporter assay was conducted. As seen in Fig. 3c, L-Sox5 alone drasti-cally downregulated the Runx2 promoter activity whereas Sox9 upregulated it. Cotransfection of L-Sox5 and Sox9 led to a decrease in Runx2 promoter activity.

Discussion

In the present study, we investigated the role of L-Sox5 during chondrocyte differentiation by retrovirally overexpressing the gene in ATDC5 cells. We found inhibitory effects of L-Sox5 on the differentiation of ATDC5 chondroprogenitor cells.

ATDC5, a teratocarcinoma-derived cell line serves as an excellent model for studying molecular mechanisms regulating the two crucial stages of cartilage formation: early differentiation of committed stem cells and the terminal differentiation of proliferating to hypertrophic chondrocytes [8]. In this study, we analyzed the effects of L-Sox5 on chondrogenesis that involves the differentiation of committed stem cell to proliferating chondrocytes and late chondrogenesis that entails fully differentiation of proliferating chondrocytes. Although chondrogenesis occurs in a sequential and stage-specific manner, it is not clear how

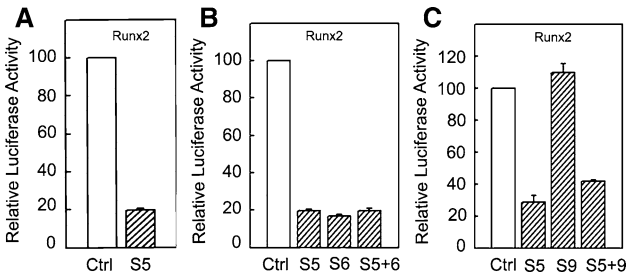

Fig. 3 Promoter activity of Runx2 regulated by L-Sox5 **a** Effects of L-Sox5 on the RUNX2 promoter activity. **b** Effects of L-Sox5 and Sox6 on the RUNX2 promoter activity. **c** Effects of L-Sox5 and Sox9 on the RUNX2 promoter activity. (S5: L-Sox5, S6: Sox6, S9: Sox9)

L-Sox5 controls the balance between early and late chondrogenesis. Here, we first demonstrated that L-Sox5 protein levels diminish along with chondrocyte differentiation whereas mRNA levels remain unaffected. None of the previous studies has reported this phenomenon although unaltered gene expression of L-Sox5 during in vitro chondrogenesis has been observed earlier in human bone marrow stem cell chondrogenic cultures [9]. Henceforth, we found a critical event regulating cartilage formation where protein expression of L-Sox5 is reduced to allow chondrogenesis with mRNA levels staying unaffected.

Sox protein expression levels have been reported to be regulated by the ubiquitin-proteasome pathway. Sox9 has been shown to be degraded by the 26S proteasome and is a specific target for the E6-AP ubiquitin ligase [10]. An E3 ligase Trip12, a HECT domain E3 ubiquitin ligase, recognizes and polyubiquitinates Sox6 [11]. Therefore, these lines of evidence indicate the possibility that L-Sox5 protein might undergo proteasome-mediated degradation to maintain the differentiation of cells toward chondrogenic lineage. Further studies that assess whether L-Sox5 protein downregulation occurs at protein synthesis or proteasome-mediated degradation level would be interesting.

During initiation of chondrogenesis, mesenchymal stem cells condense and form cartilaginous nodules, a prerequisite for cartilage formation [12]. Under influence of insulin ATDC5 cells lose contact inhibition and grow beyond confluence to produce multiple layers of cells for forming cartilaginous nodules [13]. We found that L-Sox5 delayed the differentiation of insulin-induced ATDC5 cells by attenuating the cartilage nodule formation as assessed by alcian blue staining. Furthermore, L-Sox5 inhibited the chondrocyte markers gene expression for type II Collagen, Aggrecan, Comp, Lumican, Decorin, Matrilin, and suppressed hypertrophic/osteoarthritic markers gene expression for Runx2, type X collagen, and Asporin. In line with this Decorin, Lumican, and Asporin was found to be downregulated by L-Sox5 in microarray results. Expression of Asporin is associated with osteoarthritis and that of the other extracellular proteins i.e., Osteoglycin, Osteomodulin, Periostin, and Bone Sialoprotein, that are found to be downregulated by L-Sox5 in this study might also be associated with osteoarthritis [12, 14]. Nonetheless, how these genes are regulated in the pathophysiological context of cartilage is largely unknown. Possible explanation might be that Runx2 expression and function are completely inhibited by PTHrP and is silent in chondrocytes before prehypertrophy and hypertrophy [15]. These results indicate that constitutive overexpression of L-Sox5 possibly leads to negative regulation at least in part by regulating critical fate of chondrogenesis.

It has been reported that L-Sox5 and Sox6 are functionally redundant in chondrogenesis and cartilage development [16]. Dual luciferase assay results obtained in this study clearly showed that L-Sox5 and Sox6 suppressed Runx2 promoter activity almost at same levels, further corroborating with the results from Smits et al. [16]. Furthermore, our promoter assay results demonstrated that although Sox9 alone did upregulate Runx2 promoter activity, cotransfection with L-Sox5 potently repressed Sox-9 mediated upregulation. Therefore, these results suggest that L-Sox5 function on the RUNX2 promoter might be independent of Sox9.

Our results provide first line of evidence that overexpression of L-Sox5 in ATDC5 cells reduces the chondrogenic differentiation without abolishing the commitment of cells toward chondrogenic lineage. These results indicate that L-Sox5 in ATDC5 cells might play an inhibitory role during chondrocyte maturation. This inhibitory role is mainly manifested by our observation of delayed or suppressed expression of chondrocyte marker genes. Even though similar and dissimilar regulatory mechanisms have been uncovered [3, 17, 18], it is still difficult for us to explain the inhibitory role by L-Sox5 found in this study, so more efforts are needed to uncover the mechanisms underlying cartilage-specific gene expression using our in vitro model.

In conclusion, results from our gain of function study suggest that L-Sox5 is an important regulator of chondrogenesis where it delays fully differentiation. Understanding of molecular regulatory network of L-Sox5 may prove useful in advancing cartilage tissue engineering and the data generated in this study shall aid in designing further studies to find novel genes regulated by L-Sox5 and the underlying mechanisms.

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Conflict of interest The authors declare that they have no conflicts of interests.

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Supplementary Table1. Primers used in this study

Gene	Primer sequences (5'-3')	Genbank accession no.
Gapdh	GGGCATCTTGGGCTACAC GGTCCAGGGTTTCTTACTCC	BC096590
Col2a1	TTGAGACAGCACGACGTGGAGG AGGTTGCCATCGCCATAGCTG	NM_031163
Aggrecan	GAACCTTCGCTCCAATGACTCTG AGGGAACTCGTCCTTGTCACCAT	NM_007424
Lumican	CTTGGCATTAGTCGGTAG CTCGGTCAGGTTGTTGTAG	NM_008524
Comp	AATGCGGCGCTGCAAGACG TTAGCGAAGGTCAGTCCCAC	NM_016685
Decorin	GTCCAGAGGCATTCAAAC GGATCGCAGTTATGTTGG	BC138564
Matrilin	GTGGTGACTGATGGGAGGC GCAGGCACAGGTGTAGGAG	NM_010769
L-Sox5	ACTGACCCTGATTTACCTC ACGGACTCGCCACTCTGTCG	AJ010604
Runx2	CACCATGGTGGAGATCATCG CAAGGTGAAACTCTTGCCTCG	NM_001145920
Col10a1	GAGCCAGGTCTCAATGGTCC TATGCCTGGGATCTTACAGG	NM_009925
Asporin	TACTCGAATGGTTGACCTTC TCATTCCCTTGAACGTGTCC	AF316825

Microarray analysis

For microarray analysis, ATDC5 cells overexpressing L-Sox5 and their mock control were subjected to chondrocyte differentiation for four days. Total RNA from each sample was extracted by Trizol. The microarray handling was performed by Capital Bio (Beijing, China) using 36K Mouse Genome Oligo Array from the Mouse Genome Version 4.0 of Operon, representing approximately 25,000 genes and 38,000 transcripts. Differential genes and their fold changes are listed in supplementary table 2.

Supplementary Table 2. Differentially regulated genes by L-Sox5

The genes in box are mentioned in Results and Disscussion. (red color denotes upregulated genes whereas green color indicates downregulated genes).

Name	Fold change
Angptl6	10.2998
Gsta1	8.1620
Steap1	5.8756
D5Ertd593e	5.4899
Atf3	4.8935
Gsta2	4.7722
Nt5e	4.4457
Csf1	4.4108
Trib3	4.0297
Hist1h2ao	4.0152

LOC677511	3.9854
Cyb5r1	3.9279
Nupr1	3.7241
Ifrd2	3.6283
Wtap	3.5706
Wars	3.5649
Bxdc5	3.5402
4632415L05Rik	3.5270
Baiap2l1	3.4582
Ppp3ca	3.4405
Myd116	3.4300
Clcn3	3.4228
6330407G11Rik	3.3895
Hist1h2ak	3.2521
Got1	3.2420
Cdsn	3.1607
Sfrs14	3.1500
Gpr137b	3.0774
Hist2h2ac	3.0734
Aldh1l2	3.0632
Tcte3	3.0391
LOC677006	3.0292
Spata5	3.0284
Leprotl1	2.9999
Dlx6	2.9985

Rgs2	2.9710
Hist4h4	2.8948
EG232887	2.8783
Lpl	2.8763
Hist1h2af	2.8277
Hspa9	2.8159
Rab11fip4	2.7940
Prmt3	2.7902
Det1	2.7814
Scara5	2.7321
Pno1	2.7268
Xrcc6	2.6822
Rrs1	2.6732
2410002F23Rik	2.6621
Avpi1	2.6419
Nol1	2.6256
Usp39	2.6253
BC049807	2.5678
Ugt1a6a	2.5676
Ftsj3	2.5530
Rfesd	2.5469
Pdcd2	2.5419
1810032O08Rik	2.5264
Nfe2l1	2.5041
Cebpb	2.5020

Usp1	2.4967
2310073E15Rik	2.4955
Drg1	2.4867
St3gal6	2.4768
Xlr3b	2.4607
Loxl4	2.4570
Crcp	2.4253
Plk4	2.4155
Ddx10	2.4064
Aldh3a1	2.4021
Nol5	2.3914
Kctd15	2.3809
Slc35e4	2.3773
2700094F01Rik	2.3757
Setd8	2.3647
Tmem183a	2.3630
Ddit3	2.3626
Tsc22d3	2.3624
Stambpl1	2.3611
Grwd1	2.3600
Dld	2.3583
Smarca5	2.3509
Gclc	2.3423
Telo2	2.3303
Retsat	2.3280

Hist2h3c2	2.3077
BC068171	2.3064
2810026P18Rik	2.3050
1500012F01Rik	2.3012
Ankrd11	2.2757
Polr3e	2.2745
Nolc1	2.2577
Cbx3	2.2570
Atp1a1	2.2553
Tnnt2	2.2521
Luc7l	2.2452
Gadd45a	2.2451
Zfp367	2.2443
Strap	2.2385
Gtpbp2	2.2334
Gtpbp4	2.2121
Uchl3	2.2009
2610204L23Rik	2.1973
Ipo11	2.1949
U2af1	2.1908
Pparg	2.1876
Cklf	2.1856
Mdn1	2.1833
Rbm4	2.1828
Trmt1	2.1740

LOC627307	2.1718
Gadd45b	2.1596
Apobec1	2.1486
Aoc3	2.1449
Farslb	2.1446
Sf3a1	2.1435
Ier5	2.1422
Cdkn2a	2.1410
Tnpo2	2.1403
Sh3bp4	2.1391
Sars	2.1343
Eprs	2.1288
3110082I17Rik	2.1119
Bloc1s2	2.1016
Cdca7	2.0999
Cdc34	2.0856
Mcm5	2.0793
Ddx21	2.0771
Psmc3ip	2.0723
Mthfd2	2.0667
LOC673987	2.0661
Sox4	2.0648
Krcc1	2.0627
Tarsl1	2.0596
Ssbp2	2.0586

Hipk1	2.0568
Srm	2.0554
Wdr77	2.0495
Rnf149	2.0383
Acot10	2.0380
Rnps1	2.0294
Otud7b	2.0243
Mrpl1	2.0234
1110049F12Rik	2.0176
Hexa	0.4997
Psmd13	0.4994
Nagk	0.4992
Pctk1	0.4982
Sumo3	0.4981
Tarbp2	0.4981
Sqle	0.4977
Ptk7	0.4968
Elovl6	0.4965
3110002H16Rik	0.4958
Plec1	0.4958
Apbb1	0.4951
Ifi203	0.4946
Rnf7	0.4944
Acat1	0.4942
Clta	0.4941

Trappc1	0.4938
Hsp90b1	0.4937
Sar1b	0.4933
Atp6ap2	0.4928
Dusp1	0.4917
Ifngr2	0.4899
Itm2c	0.4897
Xpa	0.4892
Txnl5	0.4870
Lox	0.4866
Fdft1	0.4850
Wbp5	0.4845
Gla	0.4838
Pgm3	0.4838
Ccdc125	0.4834
Daxx	0.4830
Ifi44	0.4819
Cope	0.4816
Ssr1	0.4815
Rerg	0.4807
2400001E08Rik	0.4805
Aldoa	0.4804
ENSMUSG00000052436	0.4792
Rint1	0.4791
Zfp207	0.4788

Es2el	0.4774
Stat2	0.4774
Dbn1	0.4769
Tuba2	0.4764
Aes	0.4759
Ift74	0.4759
Tusc3	0.4755
Tmsb10	0.4753
Casp6	0.4752
Rab18	0.4750
Ttl	0.4748
Olfml3	0.4729
Rin2	0.4729
Pde8a	0.4726
Slc10a7	0.4723
2310039E09Rik	0.4722
Vps53	0.4721
Khk	0.4719
Plat	0.4710
Rrbp1	0.4708
Pnrc1	0.4708
Sumo2	0.4700
Rpl41	0.4695
Vps11	0.4695
Ccne2	0.4693

Npc2	0.4688
Snx14	0.4683
Churc1	0.4678
Wdr23	0.4678
Cyp39a1	0.4674
Tapbp	0.4674
Lasp1	0.4668
9830124H08Rik	0.4659
Atp6v1e1	0.4657
Ddah2	0.4657
Fuca1	0.4654
Mxra8	0.4642
Rab3gap2	0.4611
Lamp2	0.4608
Slc25a17	0.4606
Hrasls3	0.4598
Cd1d1	0.4597
Golph3	0.4592
Sulf2	0.4591
Ypel5	0.4588
Hnrpa2b1	0.4587
Ppib	0.4582
Nanp	0.4576
Sec31a	0.4574
Dap	0.4573

Prkcdbp	0.4569
1200009F10Rik	0.4562
Spp1	0.4543
Vapa	0.4541
Ctsd	0.4539
Tssk2	0.4533
Sra1	0.4531
Csrp2	0.4527
Ext2	0.4524
Stra13	0.4518
Edem2	0.4515
Naga	0.4513
P2rxl1	0.4512
Galnt1	0.4505
Myo1b	0.4496
Oas1b	0.4492
Ppt1	0.4490
Pccb	0.4485
Hdac3	0.4475
Lrp4	0.4474
Chmp4b	0.4469
Creb3l1	0.4468
Hmgcs1	0.4463
Stambp	0.4462
Scd2	0.4462

Mboat5	0.4444
Xpnpep1	0.4438
Apoh	0.4431
Vkorc1	0.4419
Parp14	0.4419
Ckap4	0.4416
Serpinf1	0.4415
Haghl	0.4413
BC031353	0.4409
Xbp1	0.4397
4432405B04Rik	0.4385
Ndufa4	0.4377
2700060E02Rik	0.4370
Cryab	0.4368
Bsg	0.4365
Ergic3	0.4362
Stab1	0.4357
3110003A17Rik	0.4350
M6prbp1	0.4345
Armcx3	0.4345
Nfatc2	0.4342
Actr8	0.4332
Laptm4a	0.4327
Ddt	0.4325
Tsc22d1	0.4324

Prrx1	0.4315
LOC546828	0.4312
Efemp2	0.4305
Map1lc3b	0.4303
Adam15	0.4302
Tgds	0.4291
Armet	0.4286
Klhl30	0.4284
Naglu	0.4283
Ctsa	0.4280
Tmem112	0.4251
Elovl5	0.4248
Rab40b	0.4242
ENSMUSG00000059659	0.4234
Mfsd5	0.4231
Calr	0.4221
Fat1	0.4196
Nrtn	0.4183
S100a4	0.4181
Ebpl	0.4144
Tpm1	0.4143
Ifitm3	0.4124
Syngr1	0.4114
Ebp	0.4112
BC057079	0.4103

Igfbp6	0.4096
Stat1	0.4096
Leprel2	0.4090
Mdm2	0.4083
Timp2	0.4081
Urod	0.4074
Twf2	0.4072
Ptn	0.4066
Dgka	0.4061
Mylc2b	0.4060
Loxl1	0.4048
Tubb2a	0.4040
Arl1	0.4033
Cnih2	0.4023
Smpdl3a	0.4023
Dpep2	0.4021
Oas1a	0.4002
Dlx4	0.3994
Sdf2l1	0.3988
Acta2	0.3979
Dad1	0.3962
Fasn	0.3962
Gusb	0.3960
Lrrc17	0.3955
Maged1	0.3953

Fkbp1b	0.3949
Pdzrn3	0.3944
Slpi	0.3935
Ccpg1	0.3910
Col16a1	0.3908
Creb3	0.3902
Cnn3	0.3900
Oasl2	0.3892
BC051665	0.3883
Htatip2	0.3875
Arpc5	0.3869
EG317677	0.3867
Fcgrt	0.3850
Glt8d1	0.3844
Edem3	0.3843
1110005A03Rik	0.3840
Cd63	0.3837
Copz2	0.3836
Pdgfra	0.3803
Ckb	0.3798
0610007C21Rik	0.3766
9130005N14Rik	0.3733
Pcyt2	0.3727
Elovl4	0.3718
Nid1	0.3707

Ctgf	0.3701
Usp18	0.3691
Slc35a2	0.3669
Pgcp	0.3659
Arf4	0.3654
Arsa	0.3651
Ifi47	0.3649
Cmpk	0.3647
Socs1	0.3624
AK129302	0.3622
Rab3il1	0.3611
Tmem176b	0.3604
Fbln5	0.3601
Rnf31	0.3599
Pcolce	0.3585
Nipsnap3a	0.3581
Trp63	0.3581
Mpv17	0.3568
B2m	0.3565
Sar1a	0.3565
Ier3	0.3562
Cyp51	0.3556
Gamt	0.3556
Dip3b	0.3554
NP_001034298.1	0.3552

Htra1	0.3540
Crtap	0.3532
Morf4l2	0.3526
Pdia6	0.3514
Pnpla6	0.3504
Nudt2	0.3492
Cttn	0.3474
Lgals9	0.3451
Dpp7	0.3430
Asl	0.3429
Bace1	0.3425
Sdpr	0.3424
Rnf5	0.3412
Itm2b	0.3403
Cdc42ep3	0.3403
Enpp1	0.3397
Terf2ip	0.3393
Mpg	0.3367
Sparcl1	0.3331
D17Wsu104e	0.3324
Fuca2	0.3309
Gsn	0.3309
Apod	0.3302
Isgf3g	0.3293
Chchd2	0.3240

Lmcd1	0.3232
Trappc6a	0.3222
Trim25	0.3211
D11Lgp2e	0.3174
3300001P08Rik	0.3172
Tnc	0.3164
P4ha1	0.3160
Tmem176a	0.3134
Gbp3	0.3130
Maged2	0.3105
Dpysl3	0.3095
Sphk1	0.3093
Hebp1	0.3069
Tradd	0.3057
Col5a2	0.3056
Ptx3	0.3037
Triobp	0.3037
Gba	0.3030
St3gal2	0.3003
Nisch	0.2972
Cryl1	0.2970
Acads	0.2959
Adar	0.2952
Psme1	0.2944
Gm440	0.2939

Marcks	0.2939
BC029169	0.2936
Ifi30	0.2918
Gpx7	0.2899
Vcam1	0.2899
Ctsl	0.2872
Rcn1	0.2867
Rcn3	0.2855
Bst2	0.2855
BC023892	0.2853
Tgfbi	0.2838
Gstt3	0.2754
Irgm	0.2752
Fkbp11	0.2743
Plscr2	0.2725
Agt	0.2719
LOC677190	0.2690
Eno2	0.2675
Fbn1	0.2656
As3mt	0.2647
Ralgds	0.2635
Pxmp4	0.2630
Epb4.1l3	0.2591
Sparc	0.2582
Gramd3	0.2572

Iigp2	0.2564
Fmo1	0.2531
Rtp4	0.2530
Colec12	0.2526
Col1a1	0.2462
Selm	0.2435
Ctsf	0.2435
C1qtnf3	0.2431
Pld3	0.2421
Cacnb3	0.2350
Mxra7	0.2337
Gpr124	0.2329
Cyp1b1	0.2243
Pdgfrl	0.2220
Rsad2	0.2111
Psmb8	0.2051
Nid2	0.2047
Slc22a17	0.2041
Prrx2	0.2027
B230317C12Rik	0.2008
Ddit4	0.2000
Trafd1	0.1979
Mageh1	0.1976
Schip1	0.1905
Tbx6	0.1841

Selk	0.1828
Gng2	0.1814
Spon2	0.1805
Ifit1	0.1672
BC064033	0.1619
Ogn	**0.1558**
Selpl	0.1550
Tcn2	0.1503
Mgp	0.1485
Ccdc80	0.1481
Pdlim4	0.1463
Fblim1	0.1422
Cnn2	0.1401
Angptl2	0.1374
Kdelr3	0.1371
Col1a2	0.1361
Cpt1c	0.1345
Slc1a3	0.1284
Islr	0.1263
Clu	0.1257
Lsp1	0.1222
Efnb1	0.1218
Igf2	0.1203
D14Ertd668e	0.1199
Sesn1	0.1195

Ndst2	0.1151
Cd34	0.1119
Fkbp7	0.1011
Hspb1	0.1009
Tagln	0.0992
Omd	**0.0977**
Zfp354c	0.0953
Ibsp	**0.0864**
1190002H23Rik	0.0821
Mme	0.0813
Rnasel	0.0784
Tmsb4x	0.0719
Faah	0.0695
Hacl1	0.0690
Lum	**0.0659**
Igfbp5	0.0656
Wif1	0.0603
Lgals3bp	0.0587
Bhlhb2	0.0529
Dcn	**0.0497**
LOC214681	0.0461
Aspn	**0.0415**
Fah	0.0382
Olfr951	0.0372
Irf7	0.0352

Nkiras1	0.0250
Itgbl1	0.0191
Postn	**0.0095**
Penk1	0.0035