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Full Length Article

Crosstalk between Activin A and Shh signaling contributes to the proliferation and differentiation of antler chondrocytes

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ABSTRACT

Chondrocyte proliferation and differentiation are crucial for endochondral ossification and strictly regulated by numerous signaling molecules and transcription factors, but the hierarchical regulatory network remains to be deciphered. The present study emphasized the interplay of Activin A, Foxa, Notch and Shh signaling in the proliferation and differentiation of antler chondrocytes. We found that Activin A promoted chondrocyte proliferation and differentiation, and accelerated the transition of cell cycle from G1 into S phase along with the activation of Notch and Shh signaling whose blockage attenuated above function of Activin A. Inhibition of Notch pathway by DAPT led to a significant reduction in the expression of Shh signaling molecules, whereas addition of exogenous rShh rescued the delayed onset of chondrocyte proliferation and differentiation elicited by DAPT, indicating that Notch pathway is upstream of Shh signaling. Further analysis evidenced that DAPT attenuated the activation of Activin A on Shh signaling. Simultaneously, Foxa transcription factors were downstream targets of Shh signaling in chondrocyte differentiation. Moreover, Shh pathway played an important role in the crosstalk between Activin A-Notch signaling and Foxa. Collectively, Shh signaling may act downstream of Notch pathway to mediate the effects of Activin A on the proliferation and differentiation of antler chondrocytes through targeting Foxa.

1. Introduction

Chondrocyte proliferation and differentiation are two important biological events of endochondral bone formation which normally occurs in antler re-growth where chondrocytes undergo the non-cancerous rapid proliferation to drive cartilage elongation and differentiate into hypertrophic chondrocytes [1,2]. Activin A, a member of transforming growth factor β superfamily, has been recognized as a key regulator in chondrogenesis, endochondral ossification, fracture healing and bone matrix mineralization [3–7]. Deficiency of Activin A, encoded by inhibin beta A chain (Inhba) gene, led to the reduction in the proliferative and hypertrophic chondrocyte zones of tibial growth plate [8,9]. Meanwhile, Notch signaling is crucial for cartilage development and endochondral ossification due to these evidences that Notch activation induced chondrocyte differentiation in ATDC5 cells, whereas targeted disruption of recombination signal binding protein for immunoglobulin kappa J region (Rbpj)-dependent Notch signaling or deletion of mastermind like transcriptional coactivator 1 (Maml1), which is an important component of Notch signaling, may suppress chondrocyte proliferation and maturation [10–14]. But it remains unknown regarding the interplay between Activin A and Notch signaling, and their underlying molecular mechanisms in chondrocyte proliferation and differentiation.

Sonic hedgehog (Shh), a member of hedgehog family, plays a fundamental role in regulating chondrogenesis, endochondral ossification and skeleton development [15–20]. Loss of Shh in mouse embryo resulted in severe abnormalities of bone and cartilage in the cranio-facial, axial and appendicular skeleton, while forced expression of Shh in the cartilage caused craniorachischisis and joint fusion accompanied with the disorder of chondrocyte proliferation and differentiation [18–20]. Further analysis evidences that hedgehog members may bind to the transmembrane protein Patched (Ptch), release its repression on Smoothened (Smo) and lead to the activation of zinc-finger transcription factors glioma-associated oncogene homolog 1–3 (Gli1–3) that translocate into the nucleus to govern the transcription of downstream target genes [20–22]. However, little is known regarding whether Smo and Gli may mediate the effects of Shh on chondrocyte proliferation and differentiation in response to Activin A-Notch signaling.

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This study aims to determine the physiological function of Activin A, Foxa, Notch and Shh signaling in the proliferation and differentiation of antler chondrocytes, and clarify the interplay among them. The result indicates that Shh signaling may act downstream of Notch pathway to mediate the effects of Activin A on the proliferation and differentiation of antler chondrocytes through targeting Foxa.

2. Materials and methods

2.1. Tissue collection

Antler tissues are collected from three-year-old health sika deer as previously described [23] and its removal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. Briefly, the distal 5 cm of growing tip was removed and sectioned sagittally along the longitudinal axis. A part of the tip was then cut into 4–6 mm pieces, flash frozen in liquid nitrogen and stored at −80 °C for in situ hybridization, and the remaining tip was used for isolation of antler chondrocytes.

2.2. Isolation and treatment of antler chondrocytes

Antler chondrocytes were isolated by enzymatic digestion as previously described [23] and cultured with DMEM-High Glucose (Hyclone) supplemented with 10% fetal bovine serum (Life Technologie). When chondrocytes grew to 80–90% confluence, the adherent cells were digested with trypsase and inoculated into 6-well dishes at the concentration of 2×10^5 cells/well. After adherence to the culture dishes, subcultured chondrocytes were treated with recombinant human Shh protein (rShh, 25 ng/ml, R&D Systems) and recombinant human/mouse/rat Activin A protein (rActA, 100 ng/ml, R&D Systems) in the absence or presence of Smo antagonist cyclopamine (10 μM, Tocris Bioscience), Gli1 antagonist GANT58 (10 μM, Tocris Bioscience) and Notch signaling inhibitor DAPT (25 μM, Sigma), respectively. In addition, chondrocytes were alone incubated with DAPT. The rShh and rActA were dissolved in 0.5 M NaCl solution containing 0.2 mg/ml BSA or 4 mM HCl, while cyclopamine, GANT58 and DAPT were dissolved in DMSO. Controls received the vehicle only.

Table 1
The siRNAs used in this study.

Gene	Sense	Anti-sense
Shh	GCACCAUUCUCAUCAACCGTT	CGGUUGAUGAGAAUGGUGCTT
Ptch	GCAGACCAUGUUUCAGUUAT	UAACUGAAACAUGGUCUGCTT
Gli1	GCAGCUUGUGUGUAAUUAUTT	AUAAUUACACACAAGCUGCTT
Gli2	GGCUGAGGUGGUCAUCUAUTT	AUAGAUGACCACCUCAGCCTT
Gli3	CCACUUCCAAUGAUUCUUUTT	AAAGAAUCAUUGGAAGUGGTT
Foxa1	GGUCUGGGCACCAUGAAUUTT	AAUUCCAUGGUGCCCAGACCTT
Foxa2	CCCUACGCGAACAUGAACUTT	AGUUCAUGUUCGCGUAGGGTT
Foxa3	UCCUACAUCUCGCUCAUCATT	UGAUGAGCGAGAUGUAGGATT
Negative Control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Table 2
Primers for real-time PCR.

Gene	Forward Primer	Reverse primer	Size (bp)	Accession number
Shh	GTGATCCTTGCTTCCTCGCT	TGTCGGGGTTGTAATTGGGG	223	NM_009170
Ptch	GACAGCTGGGAGGAAATGCT	ACAAGGGCCACATCAAGAGG	140	NM_001205879
Smo	GTTCGGACAGACAACCCCAA	GATTCGAGTTCCGCCAGTCA	190	NM_005631
Gli1	CTGAGCCTTATGGAGCTAGA	AATGTTCAAGACGAGGACAC	207	XM_010805169
Gli2	GCACCACCCCCTCAGACTAT	AGAGTGGGGAGATGGACAGC	207	NM_001192250
Gli3	ACCATACGTCTGTGAGCACG	ATGTTTCCGGAGGGAGCTTG	160	XM_005205659
Ccna1	GTCGGACAGTAGGGTTCAGG	CTTGAGTGCGCTGGTGTCTA	145	XM_002691801
Ccnb1	TGCAACACCTGGCAAAGAATG	GCAGATAGTATTCCAAAGTTCACA	190	NM_001045872
Ccnb2	TATCAGGCGGGCAGTTTTAG	GCACTGGTTTCACAGAAGCA	158	NM_174264
Ccnd1	GCGCAGACCTTCGTTGCCCT	GCCGTTGGCGCTTCCCAGAT	123	NM_001046273
Ccnd2	AACACCGATGTGGATTGCC	GGAGAGAGCGGATTGGACG	211	NM_001076372
Ccnd3	ATTGGGAGGTGCTGGTCTTG	TGTGGCAATCATGGATGGGG	200	XM_005223490
Ccne1	ACTTCTGTACCCACACGCTG	TTGCTCGCATTTTAGGCTGC	194	XM_024978360
Cdk1	GGGTCAGCTGGCTACTCAAC	AGTGCCCAAAGCTCTGAAAA	138	NM_174016
Cdk2	GCTCACTGGCATTCCTCTTC	ACCCATCTGCGTTGATAAGC	134	NM_001014934
Cdk4	AGTGACCCTGGTGTTTGAGC	GCAGTTGGCATGAAGGAAAT	142	NM_001037594
Cdk6	TCGTGGAAGTTCAGATGTCG	TTGGTTGAGGGGATTTTGAG	128	NM_001192301
Col X	ATCCCCGGCCCAGCTGGAAT	GGGAGGCCCCTCTCACCTGG	179	NM_174634
Runx2	TCAGAACCCACGGCCCTCCC	GACAGCGGCGTGGTGGAGTG	177	XM_015459846
Alpl	GGAAGGGGGCAGGATTGAC	GGTGTACCCGCCAAAGGTAA	175	XM_015459997
Notch1	AACCGTAGCTCCTGAGAGCA	AGAGTCTGATCGTGCCCACT	116	XM_024999642
Notch2	TCGCTTCCAGTGTCTGTGTC	ACACTTTGCCCCATTCAGAC	100	XM_002686114
Notch3	TCTTATCCGGAACCGCTCTA	GAGCTCATCCACAGCATTGA	145	XM_003586246
Notch4	TTGCAGTGAGGAGGTGACAG	CACAATGGTCAATGCTGGTC	140	NM_001206948
Rbpj	GAGCGAGGGGATCAAACAGT	CAGAACAACCATCGCGTTCC	157	NM_005349
Maml1	GCTGGACTATGGCAACACCA	GGCTAGGTGCAACCAGTGAT	203	XM_024994729
Maml2	CTCACTCCCCTCCCCCATAC	CACAATGATTGCTGCCCGTG	179	NM_001098050
Maml3	CCCAGTTTCAAGGGTCTCCC	AGGTGTGTTGCTGTAAGGGG	191	XM_024977576
Foxa1	CTTTCAAGCGCAGCTATCCT	TCGCTCAGTGTGAGCATCTT	103	NM_001206029
Foxa2	TCATGTCGTCAGAGCAGCAG	CCCCTGGTAGTAGGAGGTGT	200	XM_025001047
Foxa3	GGGCTCGGTGAAGATGGAG	GTCATGTAGGAGTTGAGGGGG	120	NM_001033119
Gapdh	GAAGGGTGGCGCCAAGAGGG	GGGGGCCAAGCAGTTGGTGG	142	NM_001034034

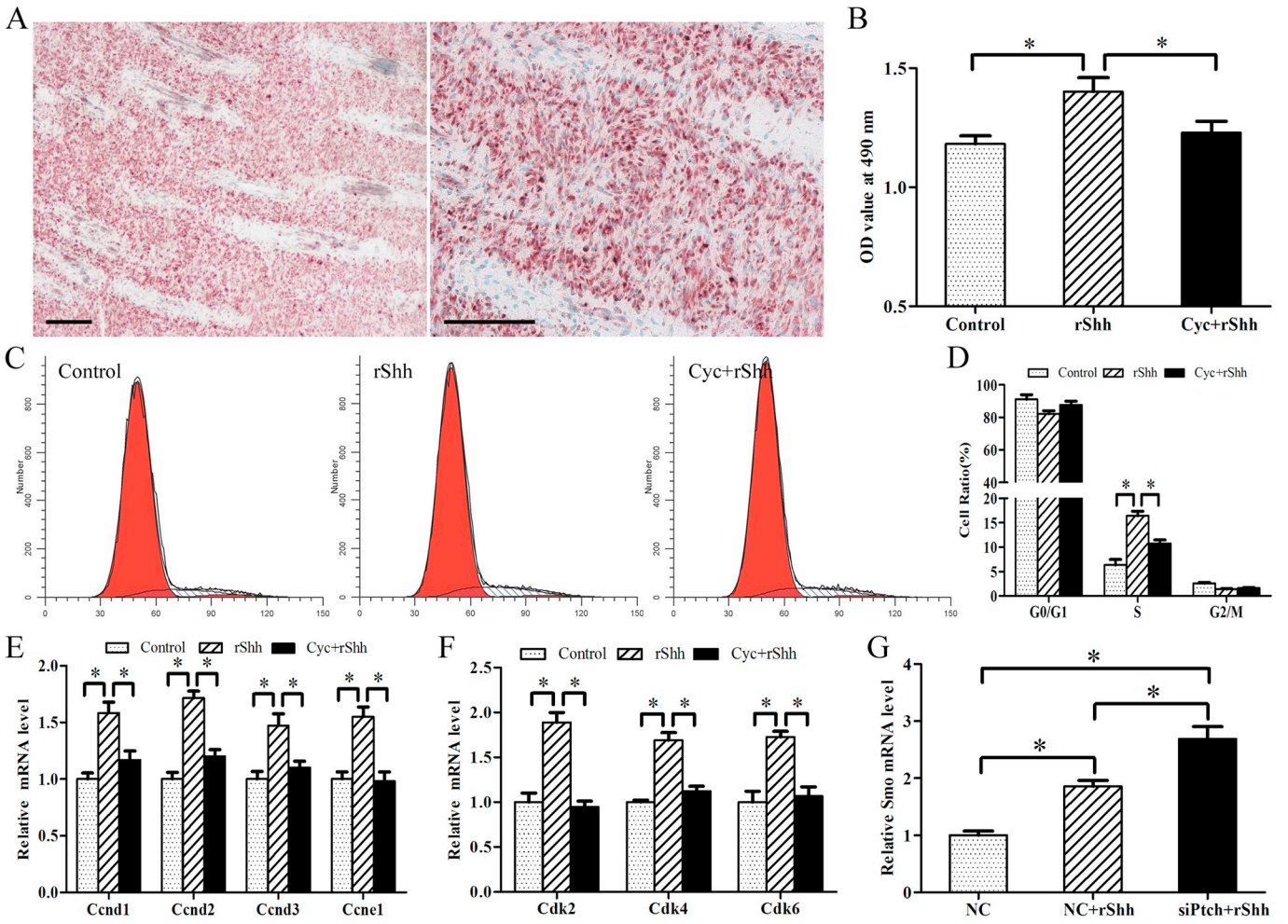

Fig. 1. Shh signaling regulates the proliferation of antler chondrocytes. A, In situ hybridization of Shh expression in antler cartilage. Bar = 60 μm. B, Effects of Shh signaling on the proliferation of antler chondrocytes. After treatment with rShh in the absence or presence of Smo antagonist cyclopamine which could also block hedgehog signaling, MTS assay was performed. Data are shown mean ± SEM. Asterisks denote significance ($P < 0.05$). Cyc, cyclopamine. C and D, Effects of Shh signaling on the cell cycle of antler chondrocytes. E, Effects of Shh signaling on the expression of Ccnd1, Ccnd2, Ccnd3 and Ccne1. F, Effects of Shh signaling on the expression of Cdk2, Cdk4 and Cdk6. G, Ptch mediated the effects of Shh on Smo expression. After transfection with Ptch siRNA and addition of rShh, the expression of Smo was determined by real-time PCR. NC, negative control; siPtch, Ptch siRNA.

2.3. In situ hybridization

Total RNA from antler tissue was reverse-transcribed and amplified with the following Shh primers: 5′-GTGAAAGCAGGTGAGGG and 5′-GACGACCGTTGCTCATTT. The amplified fragment of Shh was cloned into the pGEM-T plasmid and verified by sequencing. Shh-containing plasmid was amplified with the primers for T7 and SP6 to prepare templates for labeling. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed in vitro by a DIG RNA labeling kit (Roche Diagnostics GmbH).

Hybridization was performed as previously described using above labeled probes on 10 μm cryosections [23]. All of the sections were counterstained with 1% methyl green and the positive signal was visualized as a dark brown color. The sense probe for each gene was also hybridized and served as negative control. No detectable signals were observed with sense probes.

2.4. MTS assay

Cell proliferation was analyzed using MTS assay (Promega) in accordance with the manufacturer's protocol. Briefly, antler chondrocytes were treated for 24 h as described above, at which time 20 μl of MTS reagent was added to each well and incubated for 4 h. The absorbance was measured at 490 nm using a 96-well plate reader. Each experiment was performed in triplicate.

2.5. Flow cytometry

After antler chondrocytes were synchronized by serum starvation and subsequently treated as described above. At the end of treatment, cells were harvested by trypsinization, centrifuged, washed with PBS and then fixed overnight at 4 °C in 70% ethanol. The fixed cells were washed with PBS and stained with 0.5 ml PI/RNase staining buffer (BD Biosciences) for 15 min at room temperature. The stained cells were analyzed by flow cytometry.

2.6. RNA interference

Small-interfering RNA (siRNA) for targeting Shh, Ptch, Gli1, Gli2, Gli3, Foxa1, Foxa2 and Foxa3 as well as a scrambled siRNA (negative control) were designed and synthesized by GenePharma, and corresponding sequences were listed in Table 1. Transfection for siRNA was performed in accordance with Lipofectamine 2000 protocol (Invitrogen). After transfection with the corresponding siRNA, antler chondrocytes were collected for 24 h in the absence or presence of rShh and rActA, respectively.

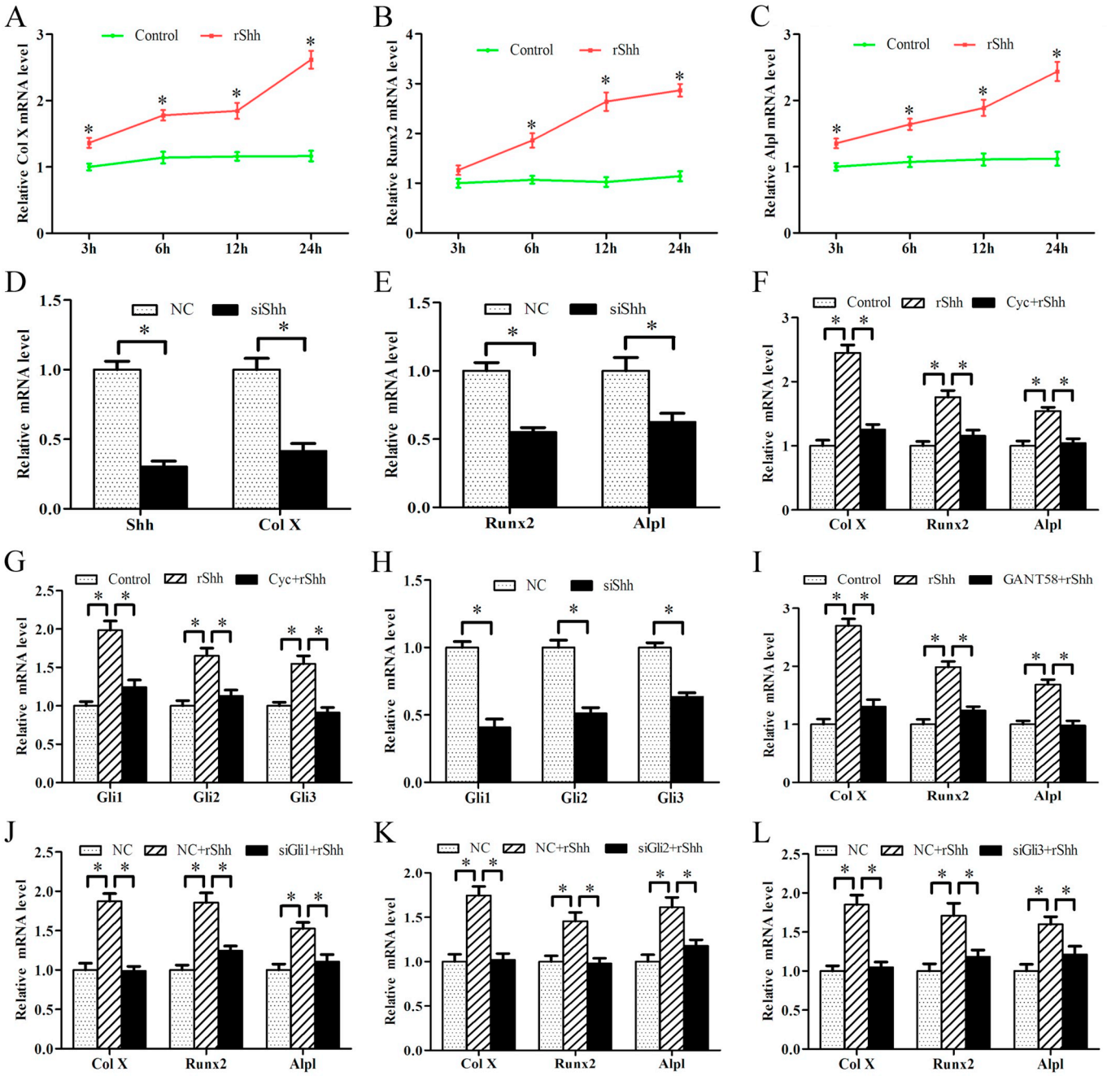

Fig. 2. Shh signaling regulates the differentiation of antler chondrocytes. A, Col X expression after treatment with rShh for 3, 6, 12 and 24 h. B, Runx2 expression after treatment with rShh for 3, 6, 12 and 24 h. C, Alpl expression after treatment with rShh for 3, 6, 12 and 24 h. D, Effects of Shh siRNA on the expression of Shh and Col X. After transfection with Shh siRNA for 24 h, the expression of Shh and Col X was determined by real-time PCR. siShh, Shh siRNA. E, Effects of Shh siRNA on the expression of Runx2 and Alpl. F, Smo mediated the effects of Shh on the expression of Col X, Runx2 and Alpl. After treatment with Smo antagonist cyclopamine and addition of rShh for 24 h, the expression of Col X, Runx2 and Alpl was determined by real-time PCR. G, Smo mediated the effects of Shh on the expression of Gli1, Gli2 and Gli3. H, Effects of Shh siRNA on the expression of Gli1, Gli2 and Gli3. I, Gli1 antagonist GANT58 abrogated the effects of Shh on the expression of Col X, Runx2 and Alpl. After treatment with Gli1 antagonist GANT58 and addition of rShh for 24 h, the expression of Col X, Runx2 and Alpl was determined by real-time PCR. J, Gli1 siRNA abolished the effects of Shh on the expression of Col X, Runx2 and Alpl. After transfection with Gli1 siRNA and addition of rShh for 24 h, the expression of Shh and Col X was determined by real-time PCR. siGli1, Gli1 siRNA. K, Gli2 siRNA blocked the effects of Shh on the expression of Col X, Runx2 and Alpl. siGli2, Gli2 siRNA. L, Gli3 siRNA abrogated the effects of Shh on the expression of Col X, Runx2 and Alpl. siGli3, Gli3 siRNA.

2.7. Real-time PCR

Total RNAs from cultured chondrocytes were extracted and then reverse-transcribed into cDNA. The expression levels of different genes were determined by real-time PCR analysis using the FS Universal SYBR Green Real Master (Roche) as previously described [23]. The result was analyzed using LightCycler 96 Software. After analysis using the $2^{-\Delta\Delta Ct}$ method, data were normalized to Gapdh expression. Primer sequences for real-time PCR were listed in Table 2.

2.8. Statistics

All the experiments were independently repeated at least three times. The significance of difference was analyzed by one-way ANOVA

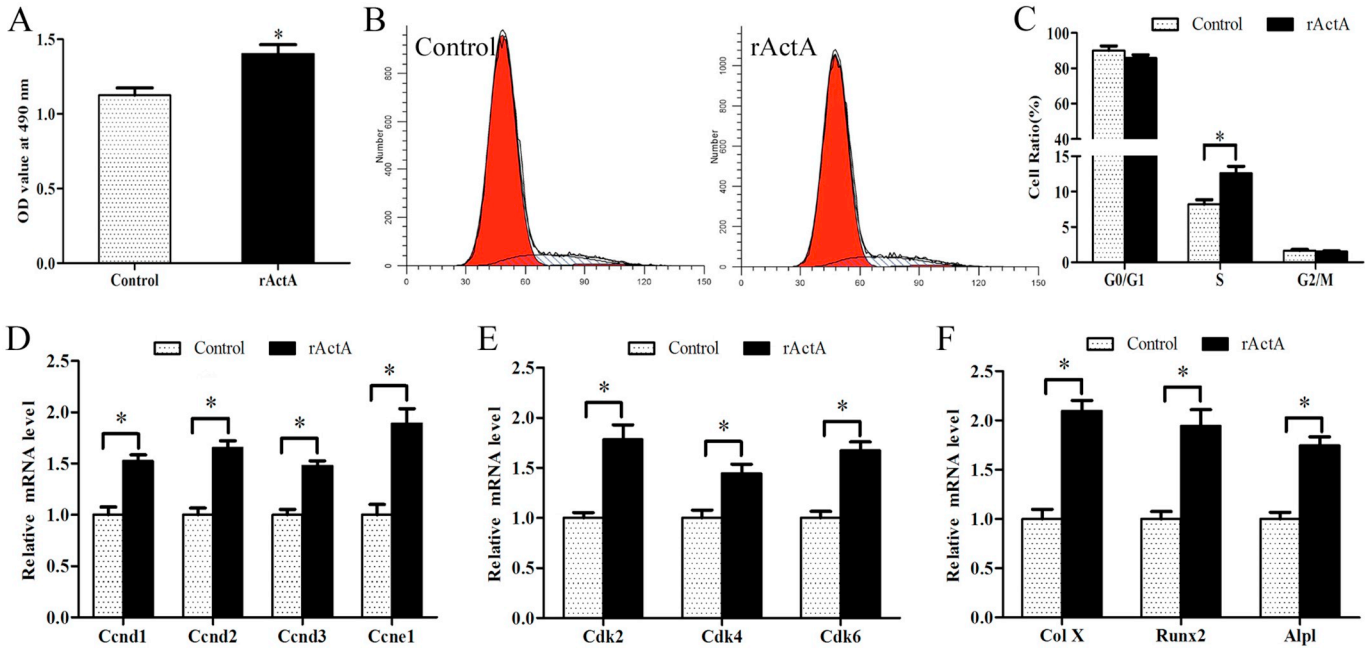

Fig. 3. Activin A regulates the proliferation and differentiation of antler chondrocytes. A, Effects of Activin A on the proliferation of antler chondrocytes. B and C, Effects of Activin A on the cell cycle of antler chondrocytes. D, Effects of Activin A on the expression of Ccnd1, Ccnd2, Ccnd3 and Ccne1. E, Effects of Activin A on the expression of Cdk2, Cdk4 and Cdk6. F, Effects of Activin A on the expression of Col X, Runx2 and Alpl.

or Independent-Samples *t*-Test using the SPSS software program (SPSS Inc., Chicago). The differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of Shh signaling on the proliferation of antler chondrocytes

To better understand the role of Shh in chondrocyte proliferation, we examined its expression in antler cartilage by in situ hybridization. The result showed that a high level of Shh mRNA signal was noted in antler chondrocytes, but there was no corresponding signal when Shh antisense probe was replaced by its sense probe (Fig. 1A). Addition of exogenous rShh boosted the proliferation of antler chondrocytes accompanied with an increase in the proportion of S-phase cells (Fig. 1B–D). To further clarify the molecular basis for the proliferative role of Shh, we analyzed its regulation on the expression of cyclin A1 (Ccna1), Ccnb1, Ccnb2, Ccnd1, Ccnd2, Ccnd3, Ccne1, cyclin-dependent kinase 1 (Cdk1), Cdk2, Cdk4 and Cdk6. The data illustrated that rShh up-regulated the expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6 in antler chondrocytes, but did not alter the expression of Ccna1, Ccnb1, Ccnb2 and Cdk1 (Fig. 1E and F).

It is well-known that binding of hedgehog to Ptch may relieve the repression of Smo and then initiate the hedgehog response [21,22]. Knockdown of Ptch by specific siRNA increased the induction of rShh on Smo (Fig. 1G). Supplementation of Smo antagonist cyclopamine, which also blocked the hedgehog signaling, attenuated the stimulation of rShh on chondrocyte proliferation and abrogated the accumulation of cells in the S phase with a simultaneous reduction in the expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6 elicited by rShh (Fig. 1B–F).

3.2. Effects of Shh signaling on the differentiation of antler chondrocytes

To determine the role of Shh signaling in the differentiation of antler chondrocytes, we assessed its influence on the expression of type X collagen (Col X), runt related transcription factor 2 (Runx2) and alkaline phosphatase (Alpl), the well-known markers for hypertrophic chondrocytes [24,25]. Administration of rShh to antler chondrocytes led to the elevated mRNA levels for Col X, Runx2 and Alpl in a time-dependent manner, whereas siRNA-mediated down-regulation of Shh exhibited the opposite effects (Fig. 2A–E). Smo antagonist cyclopamine impeded the regulation of rShh on the expression of Col X, Runx2 and Alpl (Fig. 2F). It is well established that active Smo triggers an intracellular signaling cascade leading to the activation of Gli transcription factors which are the key effectors of hedgehog signaling [19,20]. In antler chondrocytes, rShh augmented the expression of Gli1, Gli2 and Gli3, but this augmentation was abolished by Smo antagonist cyclopamine (Fig. 2G). After transfection with Shh siRNA, the levels of Gli1, Gli2 and Gli3 mRNA were obviously reduced (Fig. 2H). We next analyzed whether Gli transcription factors were necessary for Shh-mediated chondrocyte differentiation. Exposure to Gli1 antagonist GANT58 prevented the up-regulation of Col X, Runx2 and Alpl generated by rShh (Fig. 2I). Consistent with above result, silencing of Gli1, Gli2 and Gli3 by the corresponding siRNA resulted in a failure in the induction of rShh on Col X, Runx2 and Alpl (Fig. 2J–L).

3.3. Effects of Activin A on the proliferation and differentiation of antler chondrocytes

Activin A is of great importance to chondrogenesis and endochondral ossification [5–8]. In antler chondrocytes, rActA augmented cell proliferation activity and resulted in a substantial accumulation of cells in the S phase along with the elevated expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6, but the levels of Ccna1, Ccnb1, Ccnb2 and Cdk1 mRNA did not represent any statistically significant difference in comparison to the control (Fig. 3A–E). In the meantime, Activin A also facilitated the differentiation of antler chondrocytes as evidenced by the increased expression of Col X, Runx2 and Alpl (Fig. 3F).

3.4. Shh signaling mediates the effects of Activin A on the proliferation and differentiation of antler chondrocytes

To explore the relationship between Activin A and Shh signaling, we

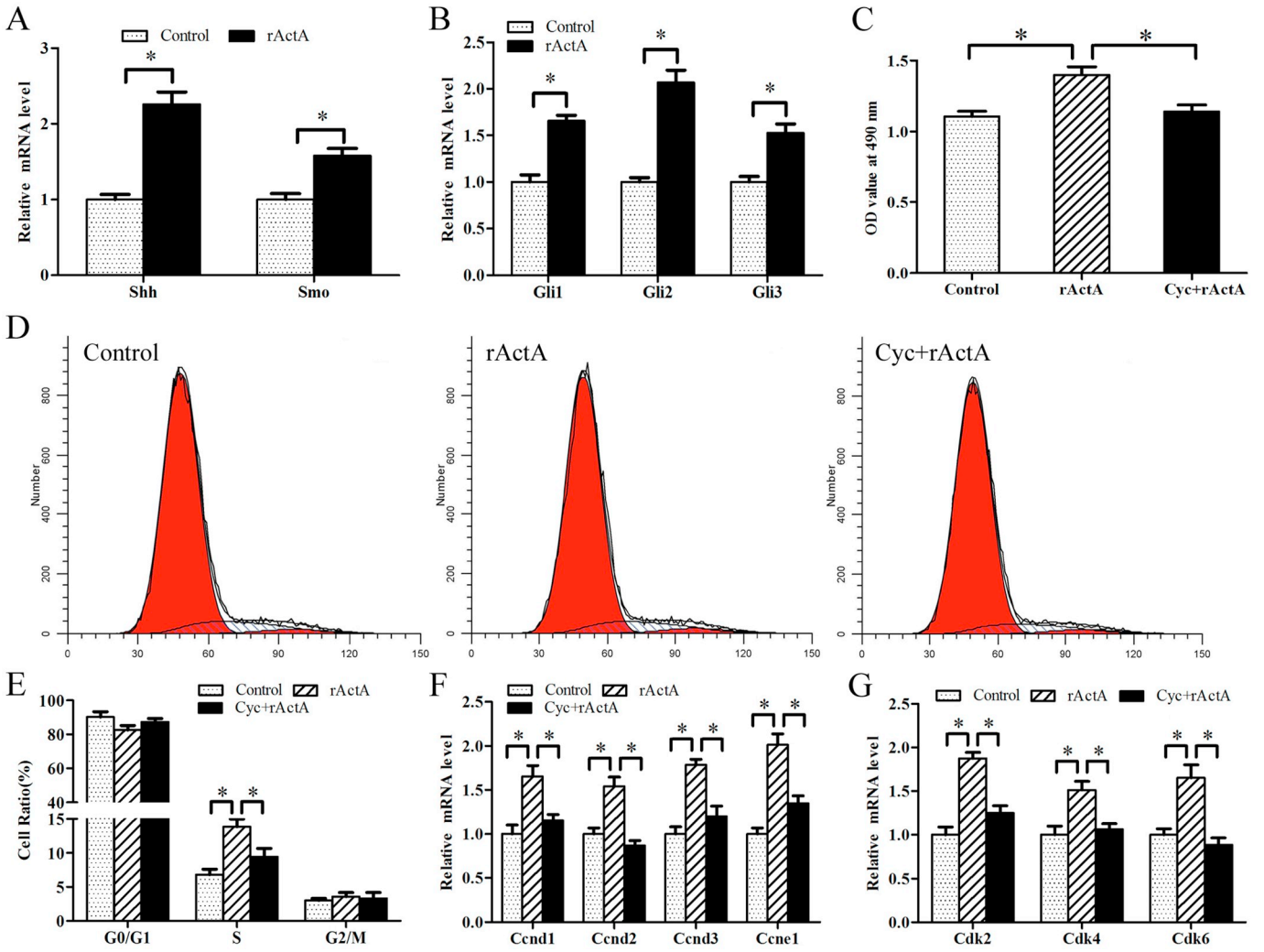

Fig. 4. Shh signaling mediates the effects of Activin A on the proliferation of antler chondrocytes. A, Effects of Activin A on the expression of Shh and Smo. B, Effects of Activin A on the expression of Gli1, Gli2 and Gli3. C, Shh signaling mediates the effects of Activin A on the proliferation of antler chondrocytes. After treatment with cyclopamine and addition of rActA, MTS assay was performed. D and E, Shh signaling mediates the effects of Activin A on the cell cycle of antler chondrocytes. F, Shh signaling mediates the effects of Activin A on the expression of Ccnd1, Ccnd2, Ccnd3 and Ccne1. G, Shh signaling mediates the effects of Activin A on the expression of Cdk2, Cdk4 and Cdk6.

investigated the regulation of Activin A on Shh signaling molecules. In antler chondrocytes, rActA raised the expression of Shh, Smo, Gli1, Gli2 and Gli3 (Fig. 4A and B), implying that Shh signaling may be downstream of Activin A. To address this, we treated antler chondrocytes with cyclopamine along with the addition of rActA, and then analyzed the role of Shh signaling in Activin A-induced chondrocyte proliferation. The results indicated that cyclopamine attenuated the stimulation of rActA on the proliferation of antler chondrocytes and expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6, and slowed the transition of cell cycle from the G0/G1 stage into the S phase elicited by rActA (Fig. 4C–G).

To elucidate whether Shh signaling exerted a role in Activin A-mediated chondrocyte differentiation, we first transfected antler chondrocytes with Shh siRNA, added the rActA and analyzed the expression of Col X, Runx2 and Alpl. The result demonstrated that knockdown of Shh removed the up-regulation of Col X, Runx2 and Alpl by rActA together with a reduction of Smo whose blockage also generated the similar action (Fig. 5A–C). Additionally, administration of Shh siRNA or cyclopamine to antler chondrocytes treated with rActA led to a decline in the expression of Gli1, Gli2 and Gli3 (Fig. 5D and E). Further analysis evidenced that inhibition of Gli transcription factors by GANT58 or corresponding siRNA alleviated the induction of rActA on Col X, Runx2 and Alpl (Fig. 5F–I).

3.5. Shh signaling mediates the effects of Notch pathway on the proliferation and differentiation of antler chondrocytes

As is known, Notch signaling is a crucial regulator of cartilage development and endochondral ossification [10–14]. In antler chondrocytes, Notch1, Notch2, Rbpj, Maml1, Maml2 and Maml3 mRNAs were abundant by RT-PCR analysis, whereas Notch3 and Notch4 mRNAs were absent (data not shown). Inhibition of Notch signaling by DAPT led to an obvious decrease in the proliferation of antler chondrocytes followed by a slow progression of cell cycle from G0/G1 through S phase (Fig. 6A–C). Meanwhile, DAPT restrained the expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6 with the simultaneous decline in the mRNA levels corresponding to Shh, Smo, Gli1, Gli2 and Gli3 (Fig. 6D–G). But after treatment with exogenous rShh or Shh siRNA, the expression levels of Notch1, Notch2, Rbpj, Maml1, Maml2 and Maml3 were indistinguishable compared with the control (Fig. 6H and I). We next ascertained whether Shh could rescue the defects of chondrocyte proliferation by inhibition of Notch

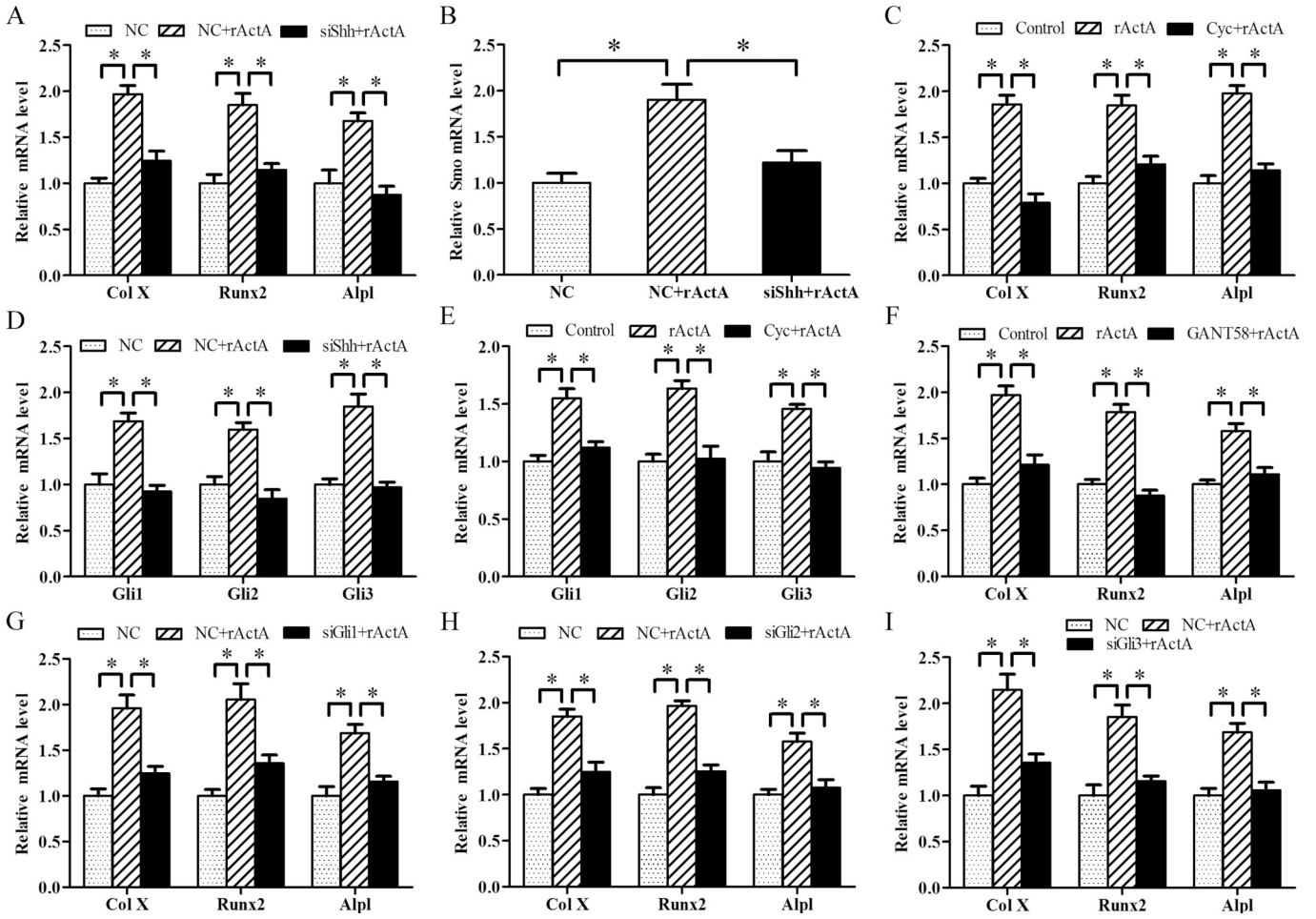

Fig. 5. Shh signaling mediates the effects of Activin A on the differentiation of antler chondrocytes. A, Shh siRNA impeded the effects of Activin A on the expression of Col X, Runx2 and Alpl. B, Shh siRNA abrogated the effects of Activin A on Smo expression. C, Smo mediated the effects of Activin A on the expression of Col X, Runx2 and Alpl. D, Shh mediated the effects of Activin A on the expression of Gli1, Gli2 and Gli3. E, Smo mediated the effects of Activin A on the expression of Gli1, Gli2 and Gli3. F, Gli1 antagonist GANT58 blocked the effects of Activin A on the expression of Col X, Runx2 and Alpl. G, Gli1 siRNA impeded the effects of Activin A on the expression of Col X, Runx2 and Alpl. H, Gli2 siRNA attenuated the effects of Activin A on the expression of Col X, Runx2 and Alpl. I, Gli3 siRNA prevented the effects of Activin A on the expression of Col X, Runx2 and Alpl.

signaling. The result indicated that rShh reversed the anti-proliferative effects of DAPT, restored cell cycle progression with an increased accumulation of cells in the S phase, and abrogated the repression of DAPT on the expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6 (Fig. 6A–E). Further evidence revealed that DAPT depressed the expression of Col X, Runx2 and Alpl, but this repression was ameliorated by the addition of rShh (Fig. 6J).

3.6. Notch pathway mediates the activation of Activin A on Shh signaling in antler chondrocytes

Because rActA promoted the expression of Notch1, Notch2, Rbpj, Maml1, Maml2 and Maml3 (Fig. 7A and B), we next unveiled the role of Notch signaling in Activin A-mediated chondrocyte proliferation and differentiation. Addition of Notch signaling inhibitor DAPT hindered the proliferation of antler chondrocytes in response to rActA (Fig. 7C). After DAPT treatment, rActA could not induce the accumulation of cells in S phase and the up-regulation of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6 (Fig. 7D–G). Simultaneously, DAPT counteracted the Activin A-induced chondrocyte differentiation as indicated by the reduced expression of Col X, Runx2 and Alpl (Fig. 7H).

As stated above, Activin A governed the Notch pathway which was upstream of Shh signaling. Based on these observations, we hypothesized that Notch pathway played a role in the crosstalk between Activin A and Shh signaling. To clarify this hypothesis, chondrocytes were treated with Notch signaling inhibitor DAPT following the addition of rActA. The result found that the expression levels of Shh, Smo, Gli1, Gli2 and Gli3 were distinctly lessened compared with rActA treatment alone (Fig. 7I and J).

3.7. Shh signaling regulates the differentiation of antler chondrocytes through Foxa

To elucidate the underlying mechanism by which Shh signaling governed chondrocyte differentiation, we analyzed its regulation on forkhead box A (Foxa) transcription factors which consisted of three members Foxa1, Foxa2 and Foxa3, and were required for chondrocyte differentiation [26]. Addition of rShh promoted the expression of Foxa1, Foxa2 and Foxa3, but this promotion was alleviated by Smo antagonist cyclopamine, while knockdown of Shh had the opposite effects (Fig. 8A and B). Meanwhile, chondrocytes also exhibited the similar effectiveness after blockage of Gli transcription factors by GANT58 or corresponding siRNA in the context of rShh (Fig. 8C–F). We next sought to determine the role of Foxa in Shh-mediated chondrocyte differentiation. After transfection with siRNAs targeting Foxa1, Foxa2 or Foxa3, rShh lost its ability to provoke the expression of Col X, Runx2

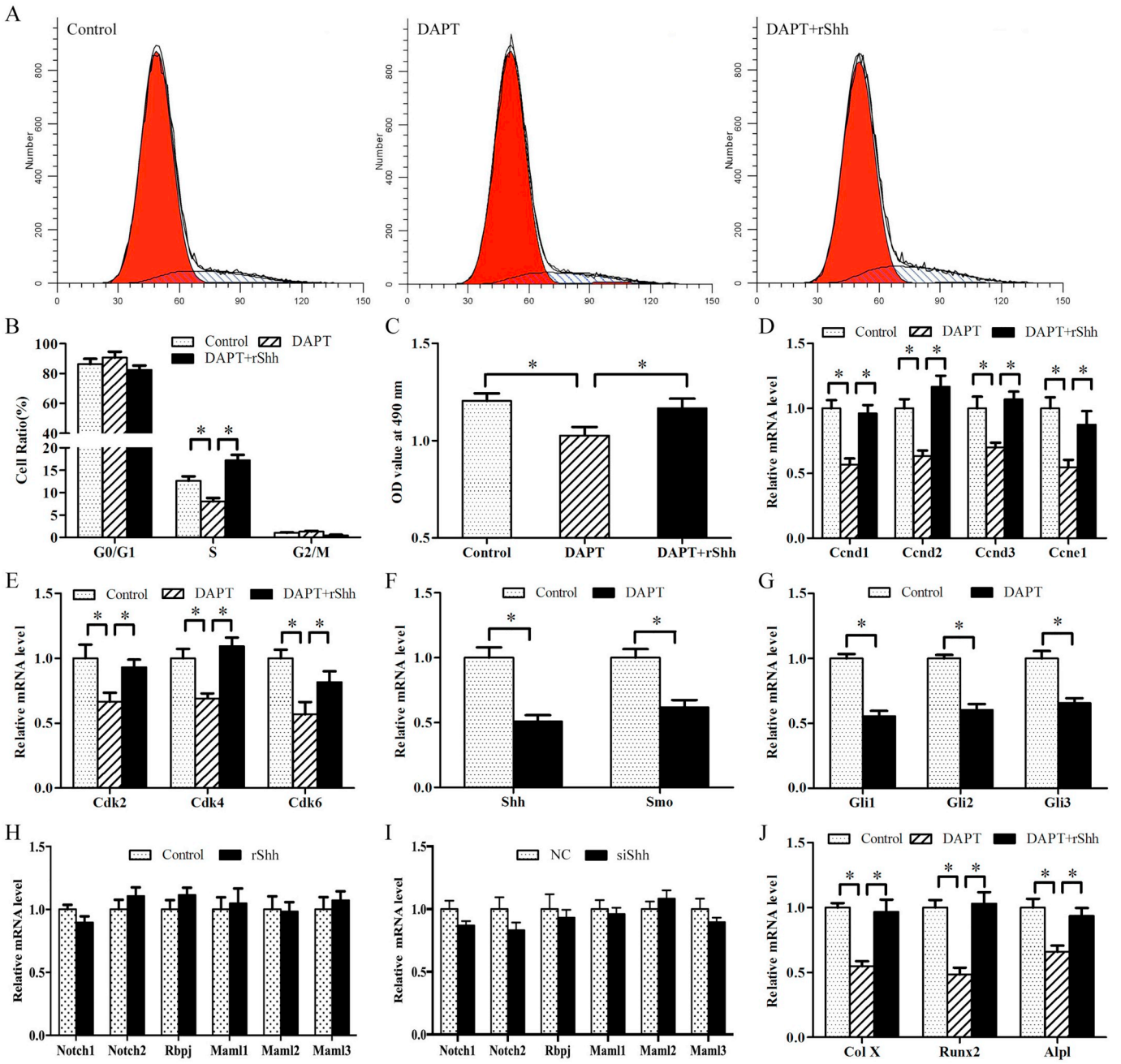

Fig. 6. Shh signaling mediates the effects of Notch pathway on chondrocyte proliferation and differentiation. A and B, Shh signaling mediated the effects of Notch pathway on the cell cycle of antler chondrocytes. After treatment with Notch signaling inhibitor DAPT in the absence or presence of rShh, flow cytometry was performed. C, Shh signaling mediated the effects of Notch pathway on the proliferation of antler chondrocytes. D, Exogenous rShh reversed the effects of DAPT on the expression of Ccnd1, Ccnd2, Ccnd3 and Ccne1. E, Exogenous rShh improved the inhibition of DAPT on the expression of Cdk2, Cdk4 and Cdk6. F, Effects of DAPT on the expression of Shh and Smo. G, Effects of DAPT on the expression of Gli1, Gli2 and Gli3. H, Effects of rShh on the expression of Notch1, Notch2, Rbpj, Maml1, Maml2 and Maml3. I, Effects of Shh siRNA on the expression of Notch1, Notch2, Rbpj, Maml1, Maml2 and Maml3. J, Exogenous rShh ameliorated the repression of DAPT on the expression of Col X, Runx2 and Alpl.

and Alpl (Fig. 8G–I).

3.8. Shh signaling acts downstream of Notch pathway to mediate the regulation of Activin A on Foxa in antler chondrocytes

As mentioned above, Activin A was upstream of Shh signaling which controlled the expression of Foxa transcription factors, implying that Shh signaling may mediate the regulation of Activin A on Foxa. To address this notion, we first analyzed the adjustment of Activin A on the expression of Foxa1, Foxa2 and Foxa3. Treatment of antler chondrocytes with rActA caused a drastic elevation in mRNA levels for Foxa1, Foxa2 and Foxa3, whose blockage attenuated the up-regulation of Col X, Runx2 and Alpl elicited by rActA (Fig. 9A–D). We next unraveled whether the regulation of Activin A on Foxa was mediated by

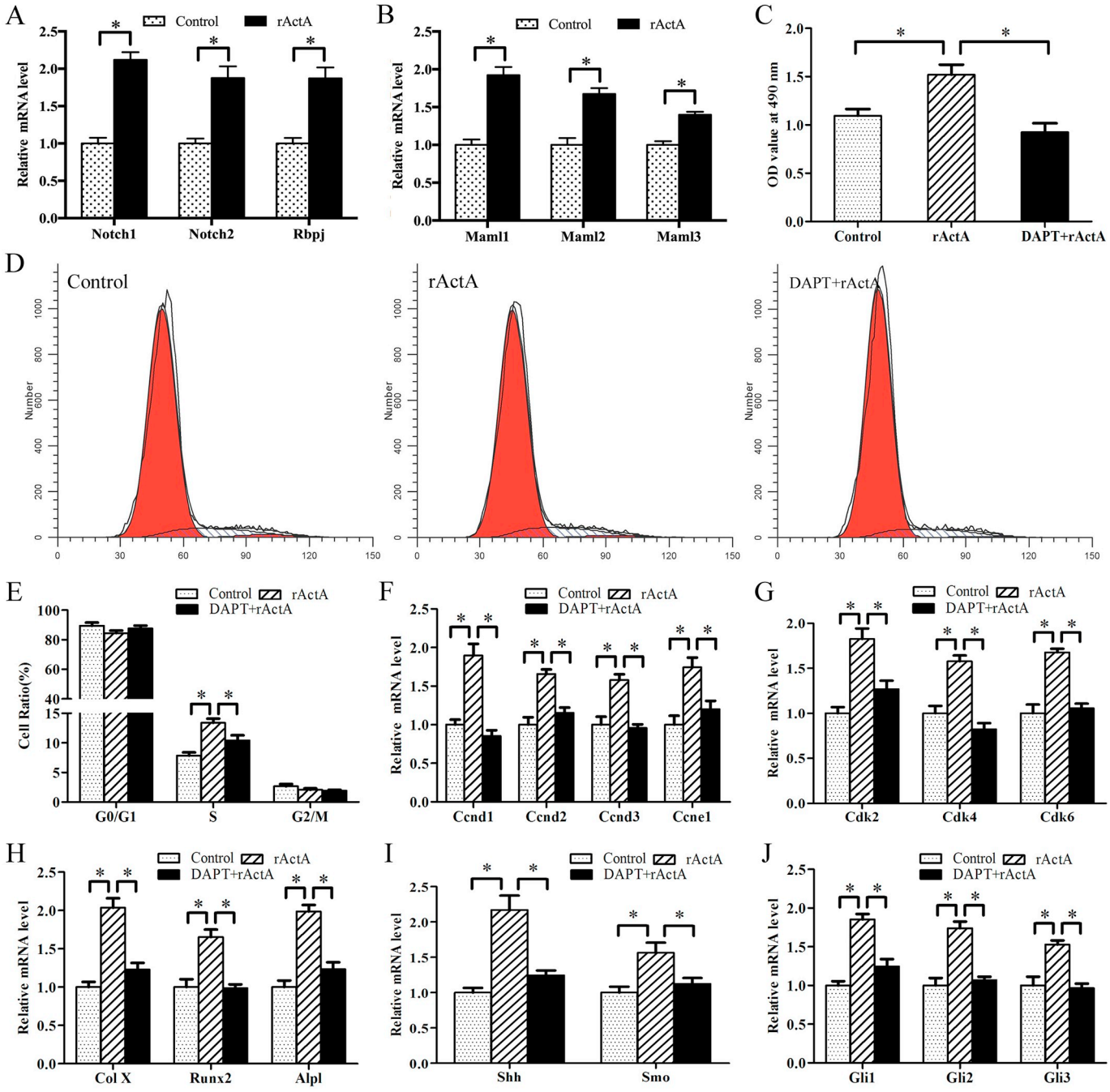

Fig. 7. Notch pathway mediates the regulation of Activin A on Shh signaling in antler chondrocytes. A, Effects of Activin A on the expression of Notch1, Notch2 and Rbpj. B, Effects of Activin A on the expression of Maml1, Maml2 and Maml3. C, Notch signaling mediated the effects of Activin A on the proliferation of antler chondrocytes. After treatment with DAPT and addition of rActA, MTS assay was performed. D and E, Notch signaling mediated the effects of Activin A on the cell cycle of antler chondrocytes. F, Notch signaling inhibitor DAPT blocked the effects of Activin A on the expression of Ccnd1, Ccnd2, Ccnd3 and Ccne1. G, DAPT hindered the effects of Activin A on the expression of Cdk2, Cd4 and Cdk6. H, DAPT prevented the effects of Activin A on the expression of Col X, Runx2 and Alpl. I, DAPT abolished the effects of Activin A on the expression of Shh and Smo. J, DAPT impeded the effects of Activin A on the expression of Gli1, Gli2 and Gli3.

Shh signaling. After transfection with Shh siRNA or addition of Smo antagonist cyclopamine, the expression of Foxa transcription factors was markedly abated in rActA-treated chondrocytes (Fig. 10A and B). In line with above findings, repression of Gli1, Gli2 and Gli3 by GANT58 or corresponding siRNA antagonized the induction of rActA on Foxa1, Foxa2 and Foxa3 (Fig. 10C–F).

As described above, Notch signaling was upstream of Shh pathway. In antler chondrocytes, Notch signaling inhibitor DAPT down-regulated the expression of Foxa1, Foxa2 and Foxa3, but the down-regulation was rescued by the supplementation of exogenous rShh (Fig. 10G and H). Additionally, we also ascertained whether Notch signaling could play a role in mediating the regulation of Activin A on Foxa transcription factors. Inhibition of Notch signaling by DAPT abrogated the induction of rActA on the expression of Foxa1, Foxa2 and Foxa3 (Fig. 10I).

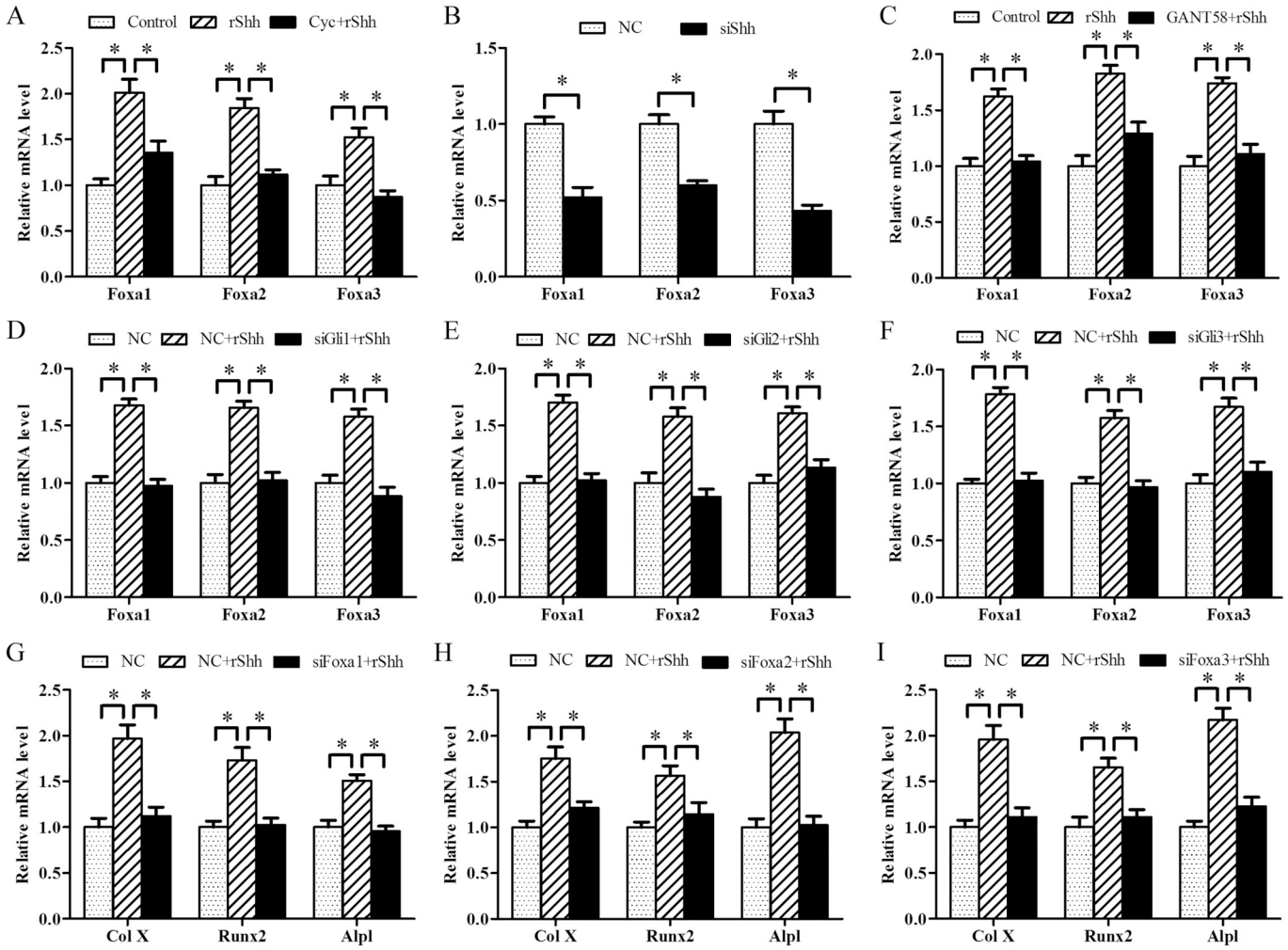

Fig. 8. Shh signaling regulates the differentiation of antler chondrocytes through Foxa. A, Shh regulates the expression of Foxa1, Foxa2 and Foxa3 via Smo. B, Effects of Shh siRNA on the expression of Foxa1, Foxa2 and Foxa3. C, Gli1 antagonist GANT58 blocked the effects of Shh on the expression of Foxa1, Foxa2 and Foxa3. D, Gli1 siRNA abrogated the regulation of Shh on the expression of Foxa1, Foxa2 and Foxa3. E, Gli2 siRNA abolished the regulation of Shh on the expression of Foxa1, Foxa2 and Foxa3. F, Gli3 siRNA abrogated the regulation of Shh on the expression of Foxa1, Foxa2 and Foxa3. G, Foxa1 mediated the effects of Shh on the expression of Col X, Runx2 and Alpl. siFoxa1, Foxa1 siRNA. H, Foxa2 mediated the effects of Shh on the expression of Col X, Runx2 and Alpl. siFoxa2, Foxa2 siRNA. I, Foxa3 mediated the effects of Shh on the expression of Col X, Runx2 and Alpl. siFoxa3, Foxa3 siRNA.

4. Discussion

Chondrocyte proliferation and differentiation are required for endochondral ossification which is a key event affecting several aspects of skeletogenesis and also involved in fracture healing, osteoarthritis and skeletal dysplasia [1,27–29]. Despite increasing knowledge of the wide range of signaling molecules and transcription factors involved in these events [21,30], the hierarchical landscape of molecular regulatory network remains largely unclear. In the present study, we provided evidence unraveling the interplay of Activin A, Foxa, Notch and Shh signaling in the proliferation and differentiation of antler chondrocytes.

As a secreted signaling factor, Shh induced chondrocyte proliferation, which was emphasized by intraperitoneal injection of BrdU in transgenic mice expressing human Shh gene in cartilage [19], and accelerated the transition of cell cycle from G1 stage into S phase. It has been documented that D-type cyclins, including Ccnd1, Ccnd2 and Ccnd3, are well-known regulators of mammalian cell proliferation and facilitate cell entry into the S phase in association with Cdk4 or Cdk6 [31,32]. Meanwhile, Ccne1 is involved in the G1/S phase transition in association with Cdk2 [31,32]. In this respect, we found that Shh could markedly increase the expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk2, Cdk4 and Cdk6. Following this initial proliferation, chondrocytes begin to differentiate into hypertrophic chondrocytes that are characterized by the elevated expression of Col X, Runx2 and Alpl [24,25]. The current evidence indicates that Shh may stimulate the differentiation of antler chondrocytes. It is generally accepted that hedgehog signal transduction is mediated by two multipass transmembrane proteins, Ptch and Smo [33,34]. Ptch does not work as a functional receptor, but serves as an inhibitor of another transmembrane protein Smo which is considered as the functional receptor [20]. Binding of hedgehog to Ptch relieves Ptch's repressive effect on Smo whose removal leads to the inactivation of hedgehog signaling [21,22,34]. In antler chondrocytes, knockdown of Ptch provoked the induction of Shh on Smo. Addition of Smo antagonist cyclopamine attenuated chondrocyte proliferation and differentiation in response to Shh, suggesting a role of Smo in Shh signaling. Further analysis indicates that active Smo triggers an

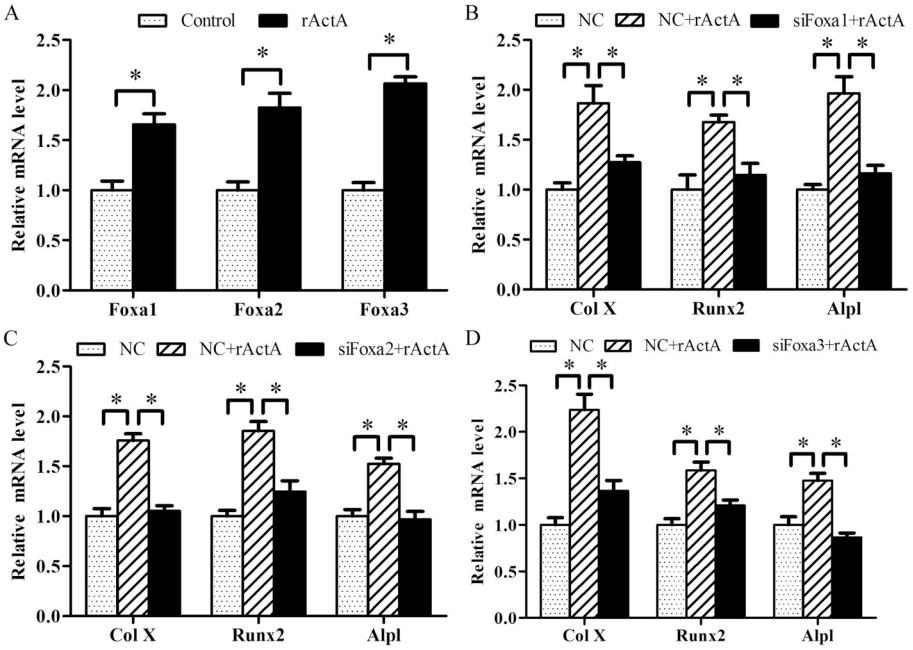

Fig. 9. Activin A regulates the differentiation of antler chondrocytes through Foxa. A, Effects of Activin A on the expression of Foxa1, Foxa2 and Foxa3. B, Foxa1 mediated the effects of Activin A on the expression of Col X, Runx2 and Alpl. C, Foxa2 mediated the effects of Activin A on the expression of Col X, Runx2 and Alpl. D, Foxa3 mediated the effects of Activin A on the expression of Col X, Runx2 and Alpl.

intracellular signaling cascade leading to the activation of Gli transcription factors which are the key effectors of hedgehog signaling [21,22]. In mammals, Gli transcription factors consist of three different members, Gli1, Gli2 and Gli3, all of which contain the activation domain at their C-terminus, whereas Gli2 and Gli3 also have an N-terminal repressor domain [21,22]. Gli1 lacks the N-terminal repressor domain and has only activator function, while analysis of Gli2 in various tissues reveals that Gli2 contains two activator domains and appears to function predominantly as a transcriptional activation [21,22]. Consistent to this notion, Gli1 and Gli2 are the positive regulators in Shh-mediated chondrocyte differentiation. By contrast, Gli3 serves primarily as a repressor responding to hedgehog signaling due to these observations that elimination of Gli3 rescues a multitude of effects in Shh or another hedgehog family member Ihh-deficient embryonic tissues including limb skeletal patterning, kidney, neural tube and cartilage development [35–38]. Interestingly, Shh up-regulated the expression of Gli3 whose blockage eliminated the induction of Shh on the expression of Col X, Runx2 and Alpl, indicating that Gli3 is a positive mediator of Shh signaling in antler chondrocyte differentiation and its function was also overlapped with Gli1 and Gli2. This concept was further supported by these findings that Gli2/3 double mutant mice exhibited more severe ventral vertebral defects than Gli2-deficient mice, while activation of Gli3 could partially rescue Gli2 mutant spinal cord defects [39,40]. After trafficking into the nucleus, Gli transcription factors may control the expression of downstream target genes. By ChIP-chip analysis, abundant Gli consensus motifs were found in the promoter region of Foxa transcription factors whose loss delayed chondrocyte hypertrophy [26,41]. In antler chondrocytes, Shh stimulated the expression of Foxa1, Foxa2 and Foxa3, but the stimulation was abrogated by the blockage of Gli1 transcription factors. Moreover, silencing of Foxa1, Foxa2 and Foxa3 led to the lack of up-regulation in mRNA levels of Col X, Runx2 and Alpl elicited by rShh, displaying that Foxa transcription factors are downstream targets of Shh signaling in chondrocyte differentiation.

Activin A was a multifunctional cytokine involved in the regulation of proliferation and differentiation of various cells, and its deletion led to the reduction in the proliferative and hypertrophic chondrocyte zones of tibial growth plate [6,8]. In pancreatic explants, Activin A raised the expression of Shh [42], which was correspondingly noted in antler chondrocytes. Meanwhile, the present study expanded the induction of Activin A on Shh signaling molecules Smo and Gli transcription factors. Blockage of Shh signaling attenuated the regulation of Activin A on chondrocyte proliferation and differentiation, indicating that Activin A is upstream of Shh signaling. This notion was potentiated by the evidence that addition of cyclopamine, which bound to Smo heptahelical bundle and was widely used to inhibit hedgehog signaling, prevented the differentiation of pancreatic tissue into intestinal structure generated by Activin A [42–45]. Further analysis demonstrates that Shh signaling may play an important role in the crosstalk between Activin A and Foxa transcription factors.

It has been well established that Notch signaling is essential for chondrocyte proliferation and differentiation. Targeted disruption of Notch signaling resulted in the delayed progression of chondrocyte maturation together with a significant decrease in BrdU-positive staining cells, whereas Notch activation promoted chondrocyte differentiation in ATDC5 cells [11–14]. Genome-wide analysis identified Shh, Smo and Gli2/3 as downstream targets of Notch1 in neural stem cells [46]. Activation of Notch led to the pronounced accumulation of Smo in NIH3T3 cells along with an increase in the levels of full-length Gli3 [47]. Consistent with above findings, inhibition of Notch signaling by DAPT also attenuated Shh pathway. Moreover, exogenous rShh rescued the delayed onset of chondrocyte proliferation and differentiation elicited by DAPT. Together these data imply that Shh signaling is downstream of Notch pathway. Simultaneously, addition of DAPT blocked the induction of Activin A on Shh signaling, indicating that Notch signaling may act as intermediate to mediate the regulation of Activin A on Shh pathway.

In summary, Shh may act downstream of Notch signaling to mediate the effects of Activin A on the proliferation and differentiation of antler chondrocytes through activation of Smo which triggers transcriptional effectors Gli and further facilitates the expression of downstream target Foxa (Fig. 11).

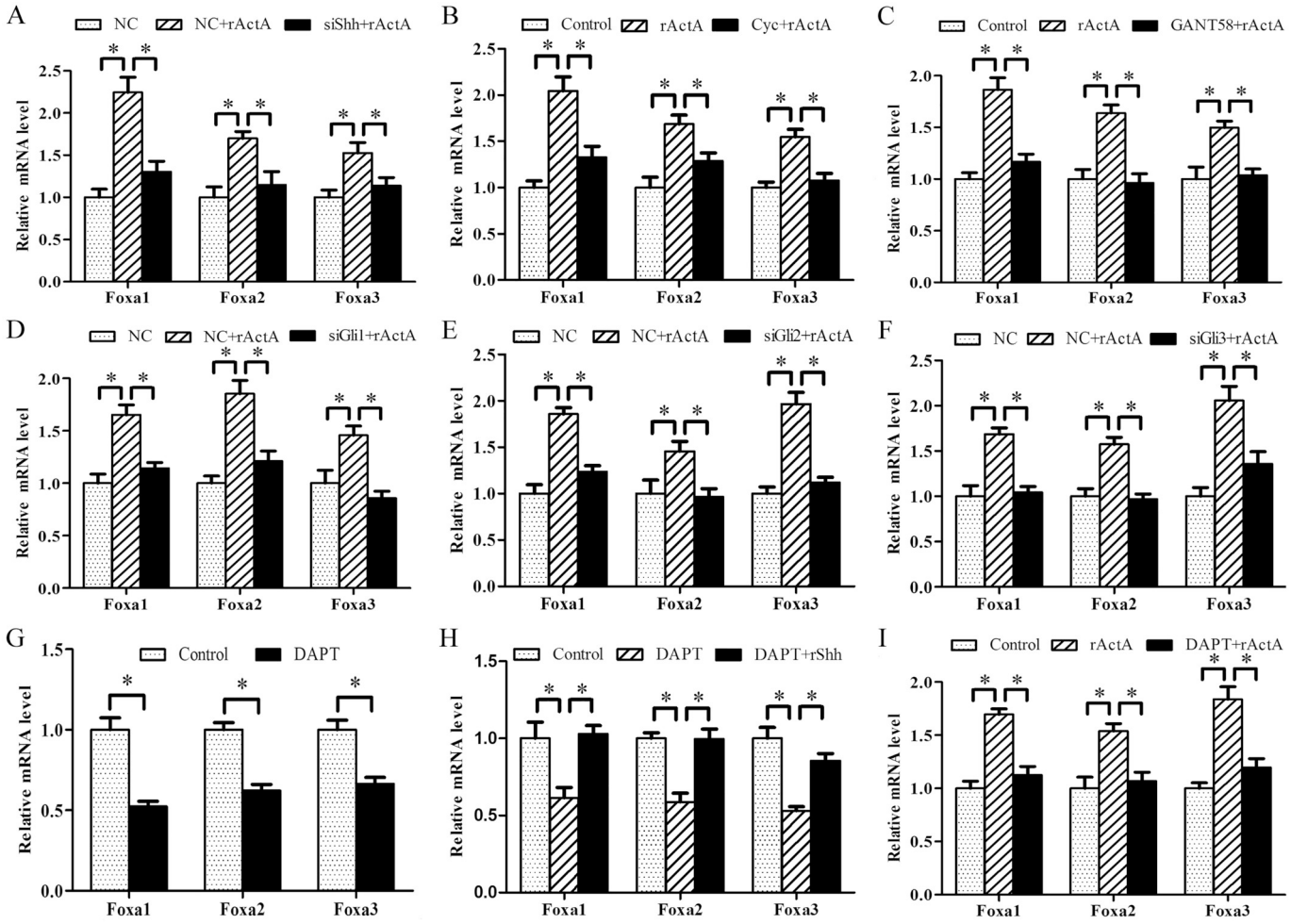

Fig. 10. Shh signaling acts downstream of Notch pathway to mediate the regulation of Activin A on Foxa. A, Shh mediated the effects of Activin A on the expression of Foxa1, Foxa2 and Foxa3. B, Smo mediated the effects of Activin A on the expression of Foxa1, Foxa2 and Foxa3. C, Gli1 antagonist GANT58 blocked the regulation of Activin A on the expression of Foxa1, Foxa2 and Foxa3. D, Gli1 siRNA impeded the regulation of Activin A on the expression of Foxa1, Foxa2 and Foxa3. E, Gli2 siRNA prevented the regulation of Activin A on the expression of Foxa1, Foxa2 and Foxa3. F, Gli3 siRNA hindered the regulation of Activin A on the expression of Foxa1, Foxa2 and Foxa3. G, Effects of Notch signaling inhibitor DAPT on the expression of Foxa1, Foxa2 and Foxa3. H, Exogenous rShh reversed the effects of DAPT on the expression of Foxa1, Foxa2 and Foxa3. I, DAPT blocked the regulation of Activin A on the expression of Foxa1, Foxa2 and Foxa3.

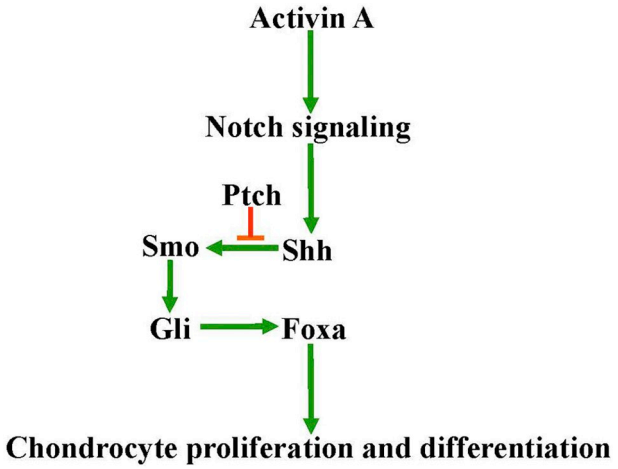

Fig. 11. Schematic diagram shows the interplay of Activin A, Foxa, Notch and Shh signaling in the proliferation and differentiation of antler chondrocytes. Activin A may provoke Notch signaling to accelerate the production of Shh. Inactivation of Ptch increases the induction of Shh on Smo, resulting in the activation of Gli transcription factors to facilitate the expression of downstream target Foxa.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.03.036.

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