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Chloride channels regulate chondrogenesis in chicken mandibular mesenchymal cells

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ABSTRACT

Voltage gated chloride channels (ClCs) play an important role in the regulation of intracellular pH and cell volume homeostasis. Mutations of these genes result in genetic diseases with abnormal bone deformation and body size, indicating that ClCs may have a role in chondrogenesis. In the present study, we isolated chicken mandibular mesenchymal cells (CMMC) from Hamburg–Hamilton (HH) stage 26 chick embryos and induced chondrocyte maturation by using ascorbic acid and β-glycerophosphate (AA-BGP). We also determined the effect of the chloride channel inhibitor NPPB [5-nitro-2-(3-phenylpropylamino) benzoic acid] on regulation of growth, differentiation, and gene expression in these cells using MTT and real-time PCR assays. We found that *CLCN1* and *CLCN3–7* mRNA were expressed in CMMC and NPPB reduced expression of *CLCN3*, *CLCN5*, and *CLCN7* mRNA in these cells. At the same time, NPPB inhibited the growth of the CMMC, but had no effect on the mRNA level of cyclin D1 and cyclin E ($P > 0.05$) with/without AA-BGP treatment. AA-BGP increased markers for early chondrocyte differentiation including type II collagen, aggrecan ($P < 0.01$) and Sox9 ($P < 0.05$), whilst had no effect on the late chondrocyte differentiation marker type X collagen. NPPB antagonized AA-BGP-induced expression of type II collagen and aggrecan ($P < 0.05$). Furthermore, NPPB downregulated type X collagen ($P < 0.05$) with/without AA-BGP treatment. We conclude that abundant chloride channel genes in CMMC play important roles in regulating chondrocyte proliferation and differentiation. Type X collagen might function as a target of chloride channel inhibitors during the differentiation process.

1. Introduction

Chloride channels (ClCs) are ubiquitously expressed in almost all eukaryotic cells and may play an important role in the regulation of intracellular pH, cell volume homeostasis, organic solute transport, and cell migration, proliferation, and differentiation.[1–4] These channels can be grouped into three subtypes and nine members according to the degree of

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Abbreviations: CMMC, chicken mandibular mesenchymal cell; AA, ascorbic acid; BGP, β-glycerophosphate; ClC, voltage gated chloride channels; *CLCN*, chloride channel gene; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; *Col2a1*, collagen type II, alpha 1, type II collagen gene; *Col10*, type X collagen gene; *Sox9*, transcription factor sox-9 gene; *CCND1*, cyclin D1; *CCNE1*, cyclin E1.

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their homology.[1] Previous studies reported that alterations and mutations of these genes caused abnormal bone modeling and altered body size. For example, loss of ClC-7 leads to osteopetrosis in mice and humans.[5] *CLCN5* gene knock mice have an abnormal dorsal spine and backward growth of teeth.[6] *CLCN3* gene knockout mice exhibit a complex phenotype including poor growth and kyphosis.[7] During embryonic development, bone development from cartilage are through endochondral and intramembranous ossification. These data indicate that chloride channel genes can regulate chondrogenesis and further affect the bone formation.

The relationship between ClCs and bone development led us to hypothesize whether ClCs contribute to chondrogenesis. During embryonic development, chondrogenesis begins with mesenchymal cell recruitment and migration, proliferation, and condensation. Many genes participate in chondrogenesis such as the nuclear transcription factor Sox9 is required for expression of the type II collagen gene, a specific marker of cartilage.[8] The aim of the present study was to characterize ClC function in chicken mandibular mesenchymal cells (CMMC). We first analyzed the expression of ClCs including *CLCN1–CLCN7* in these cells and then utilized the chloride channel blocker NPPB to assess their role in chondrocyte differentiation.

2. Materials and methods

2.1. Cell culture and treatment

CMMC were derived from mandibular processes of Hamburger–Hamilton (HH) stage 26 embryos of fertilized white leghorn chicken eggs.[9] Mandibular processes were isolated in Hanks's balanced salt solution (Sigma, St Louis, MO, USA) and collected and treated for 65 min with a mixture of trypsin and pancreatin (13:2 ratio) at a concentration of 1.5% in phosphate buffered saline (PBS) at 4 °C to facilitate the removal of the epithelia.[10] After separation of the epithelium from the mesenchymal components, cell suspension was prepared by pipeting the tissue fragments vigorously. The cells were then grown in Dulbecco's modied Eagle's medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FBS, Hyclone, Logan, CO, USA).

After the cells reached 90% confluence, the cultures were divided into four groups. The first two groups were treated with 100 μM of the chloride channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, Sigma) dissolved in dimethyl sulfoxide (DMSO, Sigma), whereas the other two groups were treated with equal volumes of DMSO as controls. One pair of the NPPB and DMSO groups was supplemented with 10 mM β-glycerophosphate (BGP, Sigma) and 150 μg/ml ascorbic acid (AA, Sigma).

2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

CMMC were seeded at a density of 8000 cells/well in 96-well plates (Corning-Costar Corp., Corning, NY, USA) containing 200 μl of DMEM/F-12 and grown overnight. The medium was replaced with NPPB and/or AA-BGP-containing medium, and the cells were cultivated for 2, 4, 6, and 8 days, respectively. The medium was changed every 2 days. At the end of the experiments, the culture medium was replaced

Table 1 – PCR primers used to detect gene expression.

Gene		Sequences (5′–3′)	Accession no.	PCR products size (bp)
Col2a1	Fwd	AGCACGACGTGGAGGTGGAT	NM_204426	100
	Rew	CAGGTCCTGGCAGGGTTCTT		
Aggrecan	Fwd	GATCTCCTTGCCCAATTACCC	XM_001232949.1	113
	Rew	CCTCAATCCCATACATCACTTCAC		
Col10	Fwd	TGTCTGCCTTCACTGTCATTCTC	M13496.1	102
	Rew	TGGGTCATAGTGTTGCTGTCT		
Sox9	Fwd	AGTACCCGCATCTGCACAA	U12533.1	160
	Rew	CCTCCTGCGTGGTTGGTA		
CLCN1	Fwd	CCTGACTGTGGGTTGTGCTG	XM_425521.2	159
	Rew	AGGACTCGGAAAATGAAGGC		
CLCN3	Fwd	GCGCTGGCAGGATTAATTGA	XM_420400.2	166
	Rew	GCTCTGCCCAAGTTTTCCAC		
CLCN4	Fwd	TGCCTTCACACTGAGGTCCA	XM_425575.2	126
	Rew	CCACCAAAGACGCCAAGAA		
CLCN5	Fwd	ATTGGGTGCGAGAGAAGTCC	XM_420265.2	184
	Rew	ATCCAGTGGGCAGAAATGTC		
CLCN6	Fwd	TTGGGAGTTGGGGGTCTTTC	XM_417644.2	188
	Rew	GCGGTACTTTGCGAGCCTCT		
CLCN7	Fwd	TACCGTGTGGTGAAGGACAA	NM_001030644.1	189
	Rew	GATCTTCACGCCATTGAGGT		
GAPDH	Fwd	TATGATGATATCAAGAGGGTAGT	K01458	199
	Rew	TGTATCCAAACTCATTGTCATAC		
CCND1	Fwd	CTGCTCAATGACAGGGTGC	NM_205381	341
	Rew	TCGGGTCTGATGGAGTTGT		
CCNE1	Fwd	GGATGGTTCCATTTGCTATGGC	NM_001031358	119
	Rew	CAAATCCAAGCTGTTTATGTGCG		