Molecular Shaping of the Beak

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Supporting online materials

Materials and Methods

Embryos. Pathogen-free fertilized chick eggs (White Leghorn) were purchased from SPAFAS (Preston, CT). Fertilized Peking duck eggs were from AA farms (Westminster, CA). Chicken embryos were staged according to Hamburger and Hamilton, 1951 (1). Duck staging was assessed by a combination of criteria including the limbs, eyes, body folds and flexures (2).

Tissue processing. In situ hybridization and immunohistochemistry were performed as described (3), and some were performed using the automated Ventana Discovery^{TM} system. Antibodies to BrdU were from Chemicon. Antibodies to collagen I were from Sigma. Cartilage and bone were stained using Alcian blue and Alizarin red, respectively. BrdU labelling was done by injecting 5 μl of 1% BrdU (Sigma) in DMEM into the vein of chicken embryos. Embryos were incubated for 1.5 hours before harvesting. After BrdU staining, slides were lightly counterstained with hemotoxylin to view all cells.

Microinjection of virus. RCAS retrovirus was prepared as described (3, 4). Stage 22-23 embryo heads were lifted using a specially designed micro-spoon. Facial prominences were identified under a dissection microscope. Virus (2 μl) was injected to beak prominences *in ovo*. Embryos were harvested from stage 35 - 37. We thank Drs. C. Tabin, P. Francis-West, and R. Johnson for RCAS viral vectors and probes.

Bead implantation. Beads (Affi-blue beads, Bio-Rad) were immersed in rhBMP4 protein (R&D) solution (100 μg/ml) for 1 hour. Albumin and other protein solutions were also used. Beads were washed in sterile DMEM and picked up with watchmakers' forceps. A tiny slit was cut in the FNM with a tungsten needle and the bead was inserted. The bead is in contact with both epithelium and mesenchyme. Stage 20 embryos were used and harvested at stage 36 - 37.

Quantification of proliferating cells. The percentage of BrdU positive cells are quantified in different grid regions of FNM by counting the number of BrdU positive cells and dividing it by the total number of cells. We adopted the grid pattern for different regions with slight modifications (5 and Dr. J. M. Richman, personal communication). The grid overlay is shown in Fig. 1B. A horizontal line was drawn across the upper margin of nasal slits. Another horizontal line was drawn to evenly bisect the area below. The upper border of the grid was then drawn to keep a similar grid height used for the lower segments. Thus there are 3 rows. The horizontal line was divided vertically into 3 equal parts. Thus there are 3 columns: left, middle, and right. In this way, the FNM is divided into 9 regions. In different stages, the sizes of these regions vary.

Three-dimensional reconstruction. 12-15 sections (42 μm between each section) were used for reconstruction. Sections were digitized using IGL Trace software (available from synapses.bu.edu/tools/trace/trace.htm). These images were aligned and rendered as a 3-dimensional view of the growth zone using Rhinoceros NURBS modeling for Windows (Robert McNeel and Associates). Only the mesenchyma cells were used. At least two embryos were used for each stage and show similar trends.

Semi-quantitative RT-PCR. To quantify BMP4 transcripts in chickens and ducks, conserved PCR primers need to be generated. We first did RT-PCR to amplify a fragment of duck BMP4 (427 bp) using the mRNA from the whole body of stage 25 ducks as a template. The primers were synthesized from chicken BMP4 mRNA sequence (Genbank accession number: X75915). Sense primer: TAACCGAATGCTGATGGTCA, Antisense primer: GCTGAGGTTGAAGACGAAGC. This duck BMP4 fragment was sequenced and aligned with chicken BMP4 to find the conserved regions to generate primers for both chickens and ducks.

Two sets of primers were generated from the conserved sequence between these two species. Set 1 (288 bp), Sense primer, CCAAAGCCATGAACTCTTGC, Antisense primer, GCTGAGGTTGAAGACGAAGC. Set 2 (188 bp), Sense primer, TACATGCTGGATCTCTACCG, Antisense primer, GCTGAGGTTGAAGACGAAGC. A set of conserved GAPDH primers were used as internal PCR controls (466 bp). Sense primer, GGCGAGATGGTGAAAGTCG. Antisense primer, CAGTTGGTGGTGCACGATG.

Stage 25 frontal nasal mass was dissected from chicken and duck embryos (2 samples each). RNA was extracted using the RNeasy kit (Qiagen). 10 μl RNA was used to conduct reverse transcription and cDNA was recovered in 40 μl Tris buffer. 2 μl cDNA was used to perform PCR (Supermix, Invitrogen). PCR conditions: 94^{o}C for 40 sec, 55^{o}C for 1 min, 72^{o}C for 1 min for 35 cycles. Both sets of BMP4 PCR primers generated similar results.

Morphometry of the beak. Beak width, depth and length were measured as shown in fig. S2D. The width of the upper beak tip was measured as shown in fig. S2E. A Student's T-test was used to compare the variation between experimental and control beaks.

fig. S1. Proliferation and molecular expression in developing chicken and duck beaks.

(A) Schematic drawing showing chicken's beak prominences at stage 23 viewed from the front. **(B)** Schematic drawing showing sagittal sections of the head at stage 20 and the frontonasal ectodermal zone (FEZ) (arrow, reference 6). **(C)** BrdU labelling at stages 26, 28, 29 and 31 (frontal sections) of chickens and ducks. Note the gradual shift of growth zones (red arrow) from the lateral to the middle regions in the chicken. In the duck, they remain as two separate zones, even at stage 31. There may be a delay in duck beak development when the same stage embryos are compared. However, complete time course analyses showed that it is a real proliferative difference, not just a relative developmental delay. **(D)** High power magnifications from region 6 of both chicken and duck FNM, stage 28. **(E)** In both chicken and duck, section in situ hybridization of BMP4 in the FNM at stage 29 show a similar pattern as BrdU positive regions. Data in other stages show a similar trend (not shown). **(F)** Whole mount in situ hybridization of BMP4 showing the duck at stage 27 has bigger MXPs with wider BMP4 expression than the chicken. FEZ, frontonasal ectodermal zone; FNM, frontonasal mass; LNP, lateral nasal prominence; MDP, mandibular prominence; MXP, maxillary prominence; oc, oral cavity; Size bar in D = 100 μm. Size bar in C, E, F = 1mm.

fig. S2. Effects of molecular perturbation and beak morphometry.

(A) Morphology of stage 36 chicken and duck beaks (side view). Alcian blue stains cartilages and Alzarin Red stains bones. **(B)** Microsurgery scheme. Regions a, b, and c in stage 20 FNM were identified. The upper nasal slit defines the upper border of region a. The lower nasal slit defines the lower border of region b. A horizontal line bisects this region into a and b. Beneath b, a similar sized region c extends toward the lower (or caudal) margin of the FNM. Region b includes the "frontonasal ectoderm zone" (6). Region a, b, or c (including both epithelia and about 10 layers of mesenchyme) was excised with a sharp tungsten needle. **(C)** Morphology of Stage 36 chicken beak treated with albumin coated beads at Stage 20 is similar to the untreated chicken beak. **(D)** Normal stage 37 chicken head. Width (blue line) and depth (brown line) of the beaks were measured at the plane along the frontal margin of the eyes. The length of the upper beak was measured from the distal tip of the maxilla to the end of the quadrato-jugal (green line). The length of the mandible was determined from the distal tip of the mandible to the end of the dentary bone (red line). **(E)** The width of the upper beak tip was measured at the widest portion of the prenasal cartilage (line), including the soft tissue. **(F)** Bar graph to show the difference of upper beak tip width (double headed arrow, fig. S2E) of stage 36 chicken and duck embryos, and chicken embryo with earlier BMP4 bead implantation. AVG±STD, n=3. mc, Meckel's cartilage; n, nasal bone; nc, nasal chonchae; pmx, premaxilla bone; pnc, prenasal cartilage. Size bar = 1mm.

fig. S3. Characterization of BMP and noggin transduced beak prominences.

(A) H&E staining of frontal sections of control, BMP4, and noggin transduced beak prominences. Note the enlarged and reduced cartilage in BMP4 and noggin specimens,

respectively. Black arrows, enlarged elements; white arrows, reduced elements. **(B)** Semi-quantitative analysis of RCAS-BMP4 viral titer versus cartilage size. When different titers of viral media were injected, there was a graded increase in cartilage size with higher titers. **(C)** BMP4 increased, whereas noggin decreased, cell proliferation. Cell proliferation (positive cells/unit area) was measured by 1.5 hr BrdU labeling. Corresponding regions from the virus injected side and the contra-lateral side (as the control) were quantified and compared. n=3. **(D)** Collagen I expression. BMP4 increased differentiation. mc, Meckel's cartilage; md, mandible; nc, nasal chonchae; oc, oral cavity; Size bar in A = 1mm. Size bar in D = 100μm.

Supporting references:

1. V. Hamburger, H. L. Hamilton, *J. Morphol.* **88**, 49 (1951).

2. R. A. Schneider, J. A. Helms, *Science* **299**, 565 (2003).

3. M. Yu, P. Wu, R. B. Widelitz, C. M. Chuong. *Nature*. **420**, 308 (2002).

4. H. Zou H, L. Niswander Science. **272**, 738 (1996).

5. M. E. MacDonald, U. K. Abbott, J. M. Richman. *Dev Dyn*. **230**, 335 (2004).

6. D. Hu, R. S. Marcucio, J. A. Helms, *Development* **130**, 1749 (2003).

Table S1. Comparison of the percentage of BrdU positive cells in different FNM regions of chicken and duck

		Chicken		Duck	
		Stage 26#	Stage 28	Stage 26	Stage 28
Left	Region 1	14.6 ± 3.6	5.9 ± 1.2	18.8 ± 2.6	9.9 ± 1.3
	Region 2	19.0 ± 2.8	11.3 ± 2.3	22.7 ± 1.6	13.9 ± 2.7
	Region 3	30.7 ± 4.8	11.5 ± 2.0	31.4 ± 3.2	24.7 ± 4.3
Middle	Region 4	9.4 ± 2.1	4.8 ± 1.1	13.3 ± 1.1	6.0 ± 2.8
	Region 5	4.9 ± 1.6	6.4 ± 1.8	13.9 ± 2.2	7.5 ± 1.9
	Region 6	17.8 ± 3.1	23.5 ± 5.1	24.0 ± 1.5	18.5 ± 2.4
Right	Region 7	14.2 ± 3.2	4.1 ± 1.0	15.3 ± 1.4	9.1 ± 2.5
	Region 8	21.7 ± 2.4	10.6 ± 3.0	19.5 ± 3.0	15.7 ± 2.4
	Region 9	29.6 ± 5.3	16.3 ± 2.0	32.2 ± 2.0	25.8 ± 5.4

* Frontal sections at the shallow level of indicated stages were used. Region 1-9 is based on MacDonald et al., 2004 (5) with modifications. Please see Fig. 1B and Materials and Methods.

Numbers shown are percentage of BrdU positive cells. Average ± standard deviation. 3 adjacent sections from the same embryo were analyzed for each value shown here. 3 independent embryos were also analyzed which show the similar trend.

fig. S1

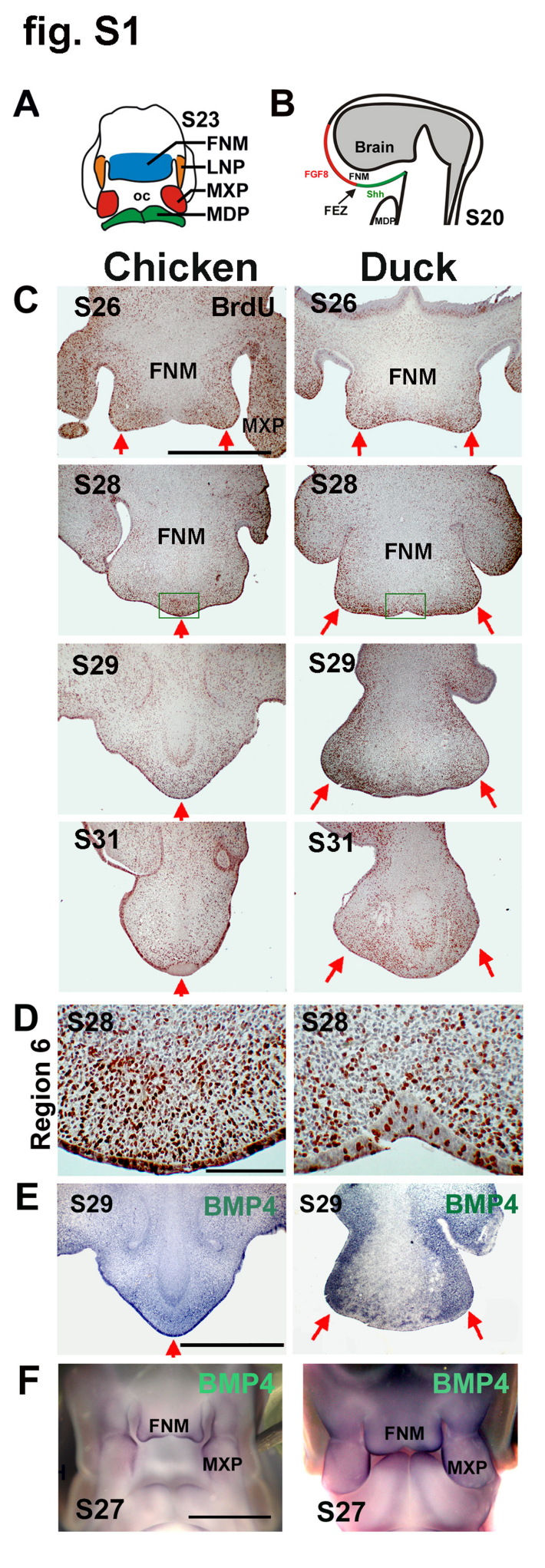

A
S23
FNM
LNP
MXP
MDP
oc
B
Brain
FGF8
FNM
Shh
FEZ
MDP
S20
Chicken
Duck
C
S26
BrdU
FNM
MXP
S26
FNM
S28
FNM
S28
FNM
S29
S29
S31
S31
D
Region 6
S28
S28
E
S29
BMP4
S29
BMP4
F
BMP4
FNM
MXP
S27
BMP4
FNM
MXP
S27

fig. S2

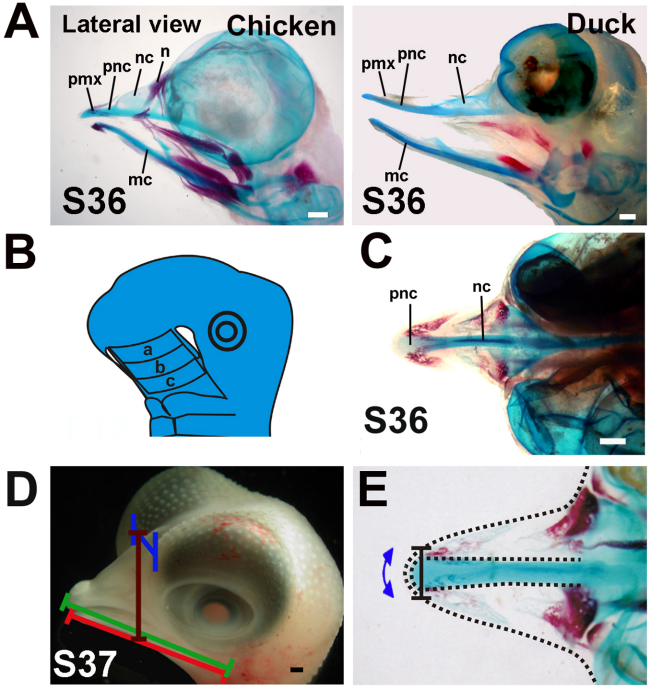
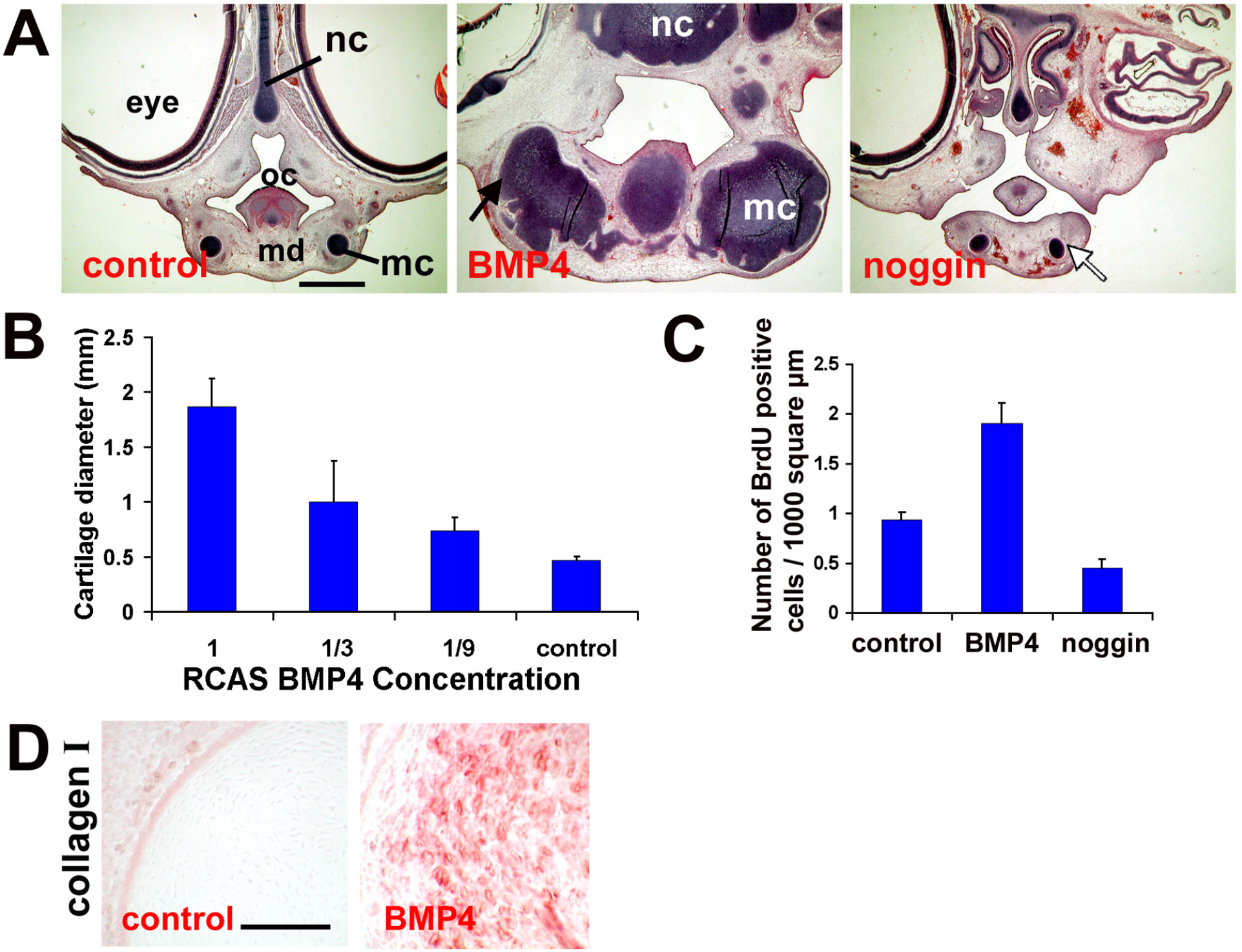

A
Lateral view
Chicken
pmx
pnc
nc
n
mc
S36
Duck
pmx
pnc
nc
mc
S36
B
a
b
c
C
pnc
nc
S36
D
S37
E
F
Beak tip width (100 µm)
16
12
8
4
0
Chicken
Duck
BMP4 bead

fig. S3


A
nc
eye
oc
md
mc
control
nc
mc
BMP4
noggin
B
Cartilage diameter (mm)
2.5
2
1.5
1
0.5
0
1
1/3
1/9
control
RCAS BMP4 Concentration
C
Number of BrdU positive cells / 1000 square µm
2.5
2
1.5
1
0.5
0
control
BMP4
noggin
D
collagen I
control
BMP4