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ARTICLE *in* DEVELOPMENTAL DYNAMICS · NOVEMBER 2001

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Connexin43 Gap Junction Protein Plays an Essential Role in Morphogenesis of the Embryonic Chick Face

IMELDA M. MCGONNELL,* COLIN R. GREEN, CHERYLL TICKLE, AND DAVID L. BECKER
Department of Anatomy and Developmental Biology, University College London, London, United Kingdom

ABSTRACT **Normal outgrowth and fusion of facial primordia during vertebrate development require interaction of diverse tissues and co-ordination of many different signalling pathways. Gap junction channels, made up of subunits consisting of connexin proteins, facilitate communication between cells and are implicated in embryonic development. Here we describe the distribution of connexin43 and connexin32 gap junction proteins in the developing chick face. To test the function of connexin43 protein, we applied antisense oligodeoxynucleotides that specifically reduced levels of connexin43 protein in cells of early chick facial primordia. This resulted in stunting of primordia outgrowth and led to facial defects. Furthermore, cell proliferation in regions of facial primordia that normally express high levels of connexin43 protein was reduced and this was associated with lower levels of *Msx-1* expression. Facial defects arise when retinoic acid is applied to the face of chick embryos at later stages. This treatment also resulted in significant reduction in connexin43 protein, while connexin32 protein expression was unaffected. Taken together, these results indicate that connexin43 plays an essential role during early morphogenesis and subsequent outgrowth of the developing chick face.**

Key words: chick; embryo; face; outgrowth; development; connexins; antisense oligodeoxynucleotides

INTRODUCTION

The developing chick face consists of a number of primordia that undergo significant morphological change to establish the basic shape of the face. This shaping process is complex with local differences in expansion mediated in part by cell proliferation (McGonnell et al., 1998). Many genes thought to be important in controlling growth of facial primordia including *Msx-1* (Mackenzie et al., 1991) and *Bmp-4* (Francis-West et al., 1994) are specifically expressed in expanding regions. However, we know little of how regionalised cell behaviour within facial primordia is co-ordinated.

Gap junctional communication has been implicated in co-ordinating patterning during vertebrate development, including facial development (Minkoff et al., 1991, 1997). Gap junctions mediate cell-cell communication by allowing direct passage of small molecules and secondary messengers (up to approximately 1 kD molecular weight) between cells (Flagg-Newton et al., 1979; Simpson et al., 1977). Gap junctions form between two adjacent cells by docking of two hemichannels (or connexons), one contributed by each cell, resulting in a continuous pore between adjacent cells. Each connexon is a hexameric unit consisting of proteins called connexins (Unwin and Zampighi, 1980). There are several types of connexin protein, commonly known by their molecular weight. Signalling through gap junction channels has been suggested to co-ordinate cell proliferation and patterning or to specify compartments in developing embryos (Becker and Mobbs, 1999; Guthrie, 1984; Lo and Gilula, 1979; Warner et al., 1984).

The chick upper beak is formed by fusion of frontonasal mass, lateral nasal processes, and maxillary primordia and is populated by ectomesenchymal neural crest from the diencephalon and mesencephalon; the lower beak is formed from paired mandibular primordia and is populated by ectomesenchymal neural crest from mesencephalon and anterior rhombencephalon (reviewed by Le Douarin and Kalcheim, 1999). Transmission electron microscopy studies have revealed local differences in abundance of gap junctions in chick maxillary primordia (Minkoff, 1983) and antibody labelling has shown that these are probably due to differential expression of connexin43 protein (Minkoff et al., 1997). In contrast, connexin32 is more evenly distributed (Minkoff et al., 1991).

Connexin43 has been implicated as the primary connexin involved in embryonic development and pattern formation (Becker et al., 1999a, Becker and Mobbs, 1999; Green et al., 1994; Lo et al., 1997; Wiens et al., 1995). Initial reports of the phenotype of mice in which connexin43 has been functionally inactivated or over-

Grant sponsor: Marsden Foundation; Grant sponsor: MRC.
Colin R. Green's present address is: Department of Anatomy, School of Medicine, University of Auckland, Auckland, Private Bag 92001, New Zealand.
Cheryll Tickle's present address is: Wellcome Trust Biocentre University of Dundee, Dow St, Dundee DD1 5EH, UK.
*Correspondence to: Imelda M. McGonnell, MCR Centre for Developmental Neurobiology, Kings College London, New Hunts House, London Bridge, London SE1 1UL, UK.
E-mail: imelda.mcgonnell@kcl.ac.uk
Received 9 April 2001; Accepted 20 July 2001

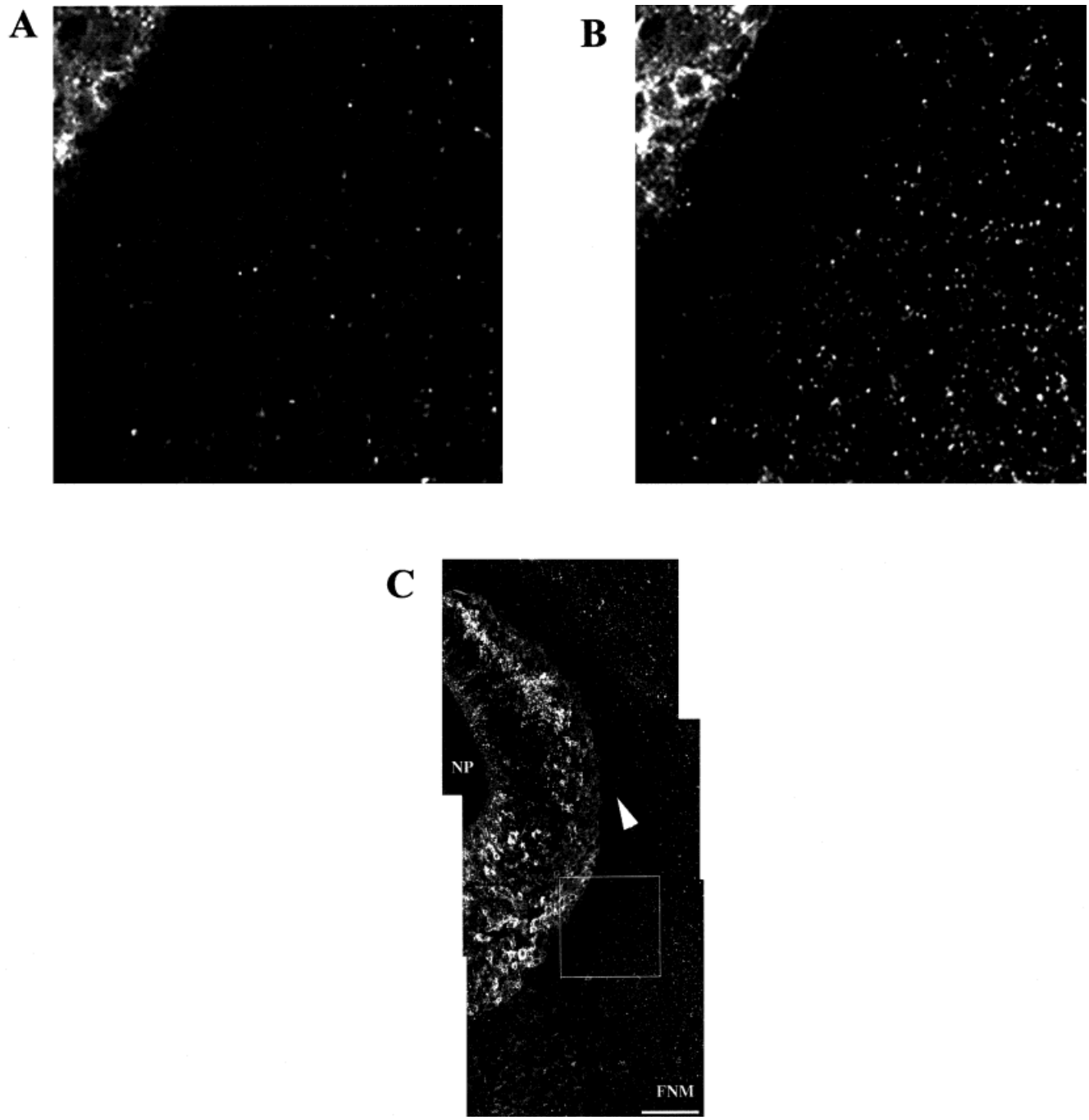


Fig. 1. Construction of images. **A:** An image of a single section from a z-series of distal nasal pit region of stage 24 chick embryo. Connexin43 positive spots can be seen in the epithelia of the nasal pit (top left) but are sparse in adjacent mesenchyme. **B:** Projection of whole z series of adjacent sections of tissue, same region as in A. This shows total number and distribution of spots of connexin43 expression in tissue 80–90 μm deep. **C:** Montage of projected z series of the entire distal nasal pit region of stage 24 chick embryo (also shown in Fig 3C, arrowhead indicates a region lacking in connexin43 protein expression). White box indicates region shown in A and B. FNM, frontonasal mass; NP, nasal pit. Scale bar = 100 μm.

expressed (Ewart et al., 1997; Reaume et al., 1995) indicated an abnormality of the cardiac outflow tract. However, more recently, abnormalities in mandibular and maxillary primordia and in the cranium have also been detected (Lecanda et al., 2000).

Here we examine expression of connexin43 and 32 gap junction proteins in the developing chick face using connexin specific antibodies. We use immunofluorescence and high magnification confocal microscopy. Under these conditions, there is punctate connexin ex-

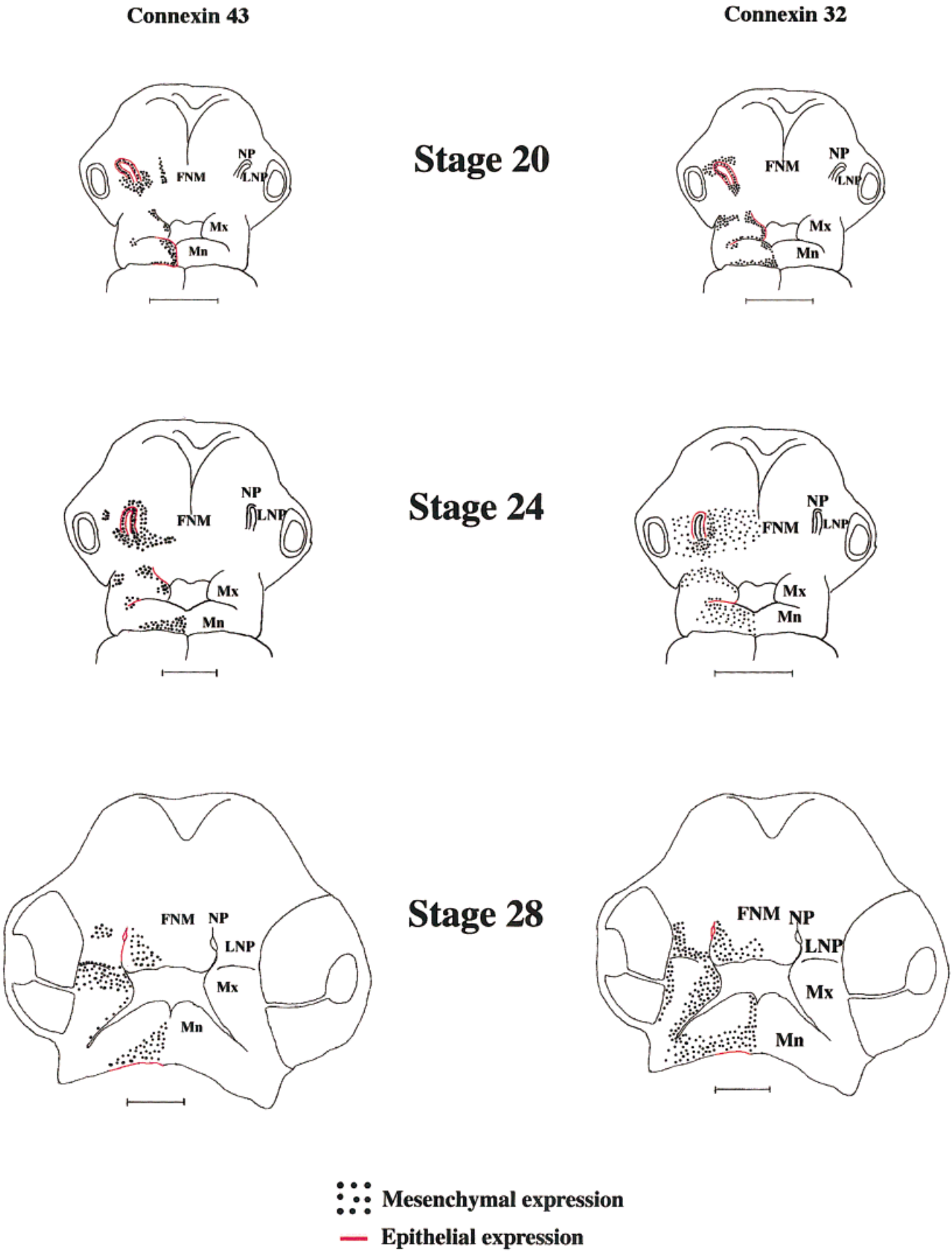


Fig. 2. Summary diagrams of distribution of connexin43 and 32 protein in chick facial primordia between stages 20 and 28. Abundant mesenchymal expression indicated by black dots, abundant epithelial expression indicated by red lines, see text for details and comparison. FNM, frontonasal mass; NP: nasal pit; LNP: lateral nasal process; Mx: maxillary primordium; Mn: mandibular primordium. Scale bar = 1,000 μm.

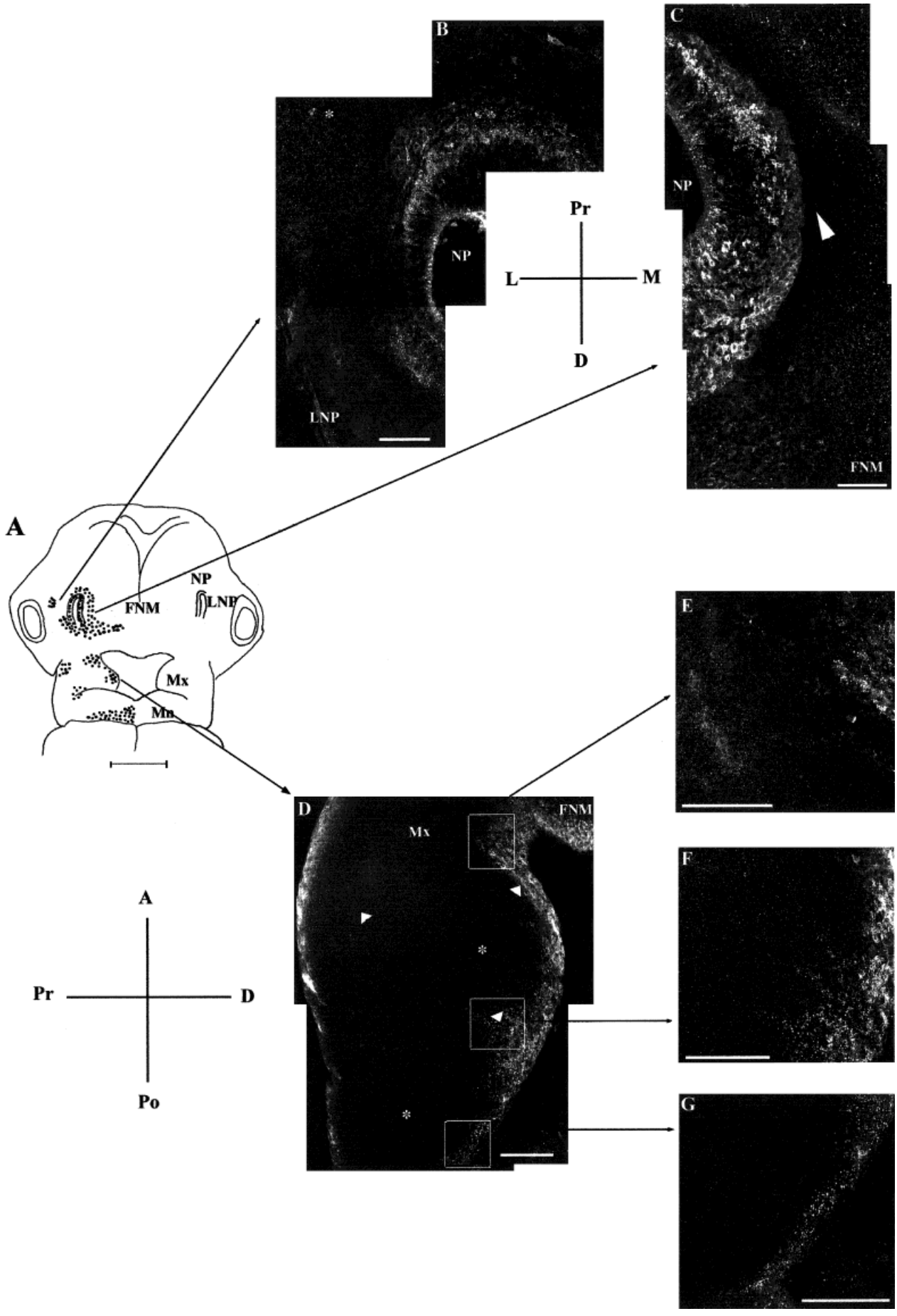


Figure 3.

pression, each spot representing a gap junction plaque (Becker et al., 1995; Green et al., 1994) (Fig. 1). Regions where we found abundant connexin43 protein are those we have previously shown to be undergoing extensive expansion and proliferation (McGonnell et al., 1998).

In order to investigate the role of connexin43 in facial development, we use an antisense approach that allows spatial and temporal reduction of connexin43 protein expression (Becker et al., 1999a) and find that connexin43 antisense treatment leads to facial defects. In order to determine mechanisms involved in this antisense perturbation of facial development, we examine patterns of cell proliferation and expression of *Msx-1* in manipulated embryos.

Facial clefting in avian and mammalian embryos can be induced by retinoic acid (Brown et al., 1997; Irving et al., 1986) and in retinoid-treated avian embryos there is decreased cell proliferation in specific cell populations in upper beak primordia (McGonnell et al., 1998). Since retinoic acid has been shown to reduce gap junction expression and communication between cells in vitro (Mehta et al., 1989; Pitts et al, 1986), we examine whether retinoic acid-induced facial defects are associated with changes in distribution of connexin43 and 32 gap junction proteins in vivo.

RESULTS

Connexin43 Protein Expression at Stages 20, 24, and 28 in the Developing Chick Face

Connexin43 immunolabelling was found throughout facial epithelia but was more abundant in surface epithelia of the nasal pit at all stages (Figs. 2, 3B,C). Levels were also elevated in proximal and distal edges and midline of mandibular primordia at stage 20; in distal anterior edges of maxillary primordia (Figs. 2, 3D) and the region where maxillary and mandibular primordia join at stage 24 (Figs. 2, 3G); and in proximal edges of mandibular primordia at stage 28 (summarised in red in Fig. 2, n = 7 for each stage).

In contrast to the largely uniform expression throughout epithelia, abundance of connexin43 protein between mesenchymal cells in different regions of the face varied considerably (summarised by black dots, Fig. 2). At stage 20, connexin43 protein was abundant in mesenchyme at distal (open) ends of the nasal pits and at stage 24 and 28 expression was also seen proximally around the nasal pit (Figs. 2, 3B,C). At stage 28 additional expression was seen in the central frontonasal mass while in mesenchyme immediately adjacent to the nasal pits, there was little connexin43 protein (Fig. 3C). At stage 20, maxillary primordia expressed connexin43 protein at anterior distal edges; at stage 24, in proximal maxillary primordium and at the join between maxillary and mandibular primordia (Figs. 2, 3D–G), and by stage 28, high levels of expression extended across anterior but not posterior maxillary primordia.

In the mandibular primordia, between stages 20 and 28, connexin43 protein was abundant at the proximal midline (Fig. 2). Transient expression was seen in the distal midline at stage 20 but there was little expression in lateral regions of mandibular primordia at any stage.

Connexin32 Protein Expression at Stages 20, 24, and 28 in the Developing Chick Face

Distribution and abundance of connexin32 protein in epithelia of chick facial primordia was more uniform than connexin43, with only surface epithelia of nasal pits (Figs. 2, 4B), distal maxillary epithelia at stage 20, and epithelia at mandibular and maxillary primordia borders at stage 24 (Fig. 4E) showing increased levels of expression (summarised in red, Fig. 2, n = 7 each stage).

In mesenchyme, connexin32 protein was also more widely distributed than connexin43 (black dots in Fig. 2). Connexin32 was expressed at high levels in mesenchyme at open ends of nasal pits (Fig. 4B), similar to connexin43. In mesenchyme adjacent to nasal pits, connexin32 was initially expressed proximally, becoming more widespread later (Figs. 2, 4B,C). Thus, expression of connexin32 and 43 proteins overlap in distal frontonasal mass and near the nasal pits at later stages (compare diagrams in Fig. 2). In maxillary primordia, connexin32 was expressed anteriorly at stage 20, in mesenchyme at borders with mandibular primordia at stage 24 (Fig. 4E), and at stage 28 concentrated distally and proximally. Thus, in anterior maxillary primordia and at mandibular-maxillary border, both connexin32 and 43 proteins are abundant (compare diagrams in Fig. 2).

In mandibular primordia, connexin32 expression was initially seen at midline and proximal edges (Fig. 2) before expanding throughout the primordium, except at proximal lateral edges (Fig. 4E–G). By stage 28, expression was restricted more proximally except at the midline (Fig. 2). Thus, connexin32 and 43 protein

Fig. 3. Connexin43 expression in stage 24 chick upper beak primordia. **A:** Diagram of chick embryo head, stage 24. Black dots summarise regions of abundant connexin43 protein in upper face as shown in B–G. **B:** Connexin43 protein abundant in nasal pit tissues and distal and proximal (*) lateral nasal process. **C:** Connexin43 protein abundant in frontonasal mass mesenchyme close to nasal pit. A region devoid of gap junctions lies immediately adjacent to nasal pit (arrowhead) **D:** Connexin43 expression abundant in epithelia at distal edge of maxillary primordium (arrowhead, top right). Abundant mesenchymal expression in anterior lateral regions (arrowhead top left) and mid distal regions (arrowhead in white box.) White boxes indicate regions magnified in E,F,G. * indicates regions of little expression. **E:** Connexin43 expression abundant in both epithelia and mesenchyme of anterior distal maxillary primordium. Note difference in appearance between epithelial and mesenchymal expression. Epithelial expression is on the right, mesenchymal on left. **F:** Connexin43 protein abundant in both epithelia and mesenchyme of mid - distal maxillary primordium. **G:** Connexin43 labelling in posterior distal maxillary primordium is predominantly in epithelia and at join between mandibular and maxillary primordia. Figure labels as in Figure 1. Axes: D: distal; Pr: proximal; M: medial; L: lateral; A: anterior; Po: posterior. Scale bar = 1,000 μm in A, 100 μm in B–G.

expression in mandibular primordia overlaps proximally and at the border with the maxillary primordia (compare diagrams in Fig. 2).

Effects of Connexin43 Antisense Oligodeoxynucleotides on Chick Facial Primordia

Embryos were treated with pluronic gel containing either connexin43 antisense oligodeoxynucleotides (ODNs) or sense ODNs or pluronic gel alone. These ODNs have previously been shown to reduce specifically connexin43 protein levels in chick embryos (Becker et al., 1999a,b). The effectiveness of this particular treatment regime in preventing connexin43 protein translation was characterised by immunohistochemisty at a series of later times (Fig. 5).

Application of connexin43 antisense ODNs to heads of chick embryos at stages 20–24 failed to significantly change levels and distribution of connexin43 protein in the face. Therefore, we tested whether adding these ODNs at earlier stages was more effective (n = 5 at each time point). To obtain a reduction in connexin43 protein in the face between stages 20 and 28, we had to treat embryos between stages 10 and 14.

In heads of embryos treated at stage 10–11, no significant changes in connexin43 protein labelling were seen 2–4 hr post treatment but after 8 hr (at stage 12) much reduced levels of connexin43 protein were noted in ectoderm, neural tube, and tissues lateral to it (Fig. 5C, compare to controls in Fig. 5B). In all control embryos (untreated, sense and pluronic gel dosed), connexin43 protein was abundant in these tissues (Fig. 5B). These lateral cells that express connexin43 protein are also HNK-1 positive and are thus of neural crest origin (Fig. 5D). A little later (at stage 14), connexin43 protein could be detected between HNK-1 positive crest cells migrating into the pharyngeal arches (Fig. 5E).

Twenty-four hours after application of antisense ODNs (embryos now at stage 17), expression of connexin43 protein in mesenchyme of frontonasal mass and 1st arch primordium (from which mandibular and maxillary primordia both derive) was still significantly reduced (Fig. 5H, compare to control in Fig. 5G), although epithelial connexin43 protein expression was observed in some embryos (Fig. 5H). Control stage 17 embryos showed abundant connexin43 expression in both epithelia and mesenchyme of facial primordia (Fig. 5G). In all treated embryos at 48 hr (stage 22), there was still little or no expression of connexin43 protein in maxillary primordia (Fig. 5L). However, expression had begun to return in distal lateral nasal processes and frontonasal mass (Fig. 5J,K) and had reached normal levels in mandibular primordia in the most embryos (Fig. 5M).

In all embryos treated between stages 12 and 14, a similar time course of connexin43 protein expression was found, with protein levels being reduced after 8 hr (stages 15–17) and remaining low in the maxillary primordium until stage 24–26.

Effect of Reduction of Connexin43 Protein Expression on Chick Facial Morphology

Forty-seven percent of surviving embryos treated with connexin43 antisense ODNs at stages 10 and 11 had detectable facial defects at stage 22 (48 hr after treatment) mostly on one side of the face (Table 1). All had defective maxillary primordium development (Fig. 6D–F, compare with controls in Fig. 6A–C), almost half had nasal pit defects (Fig. 6D,H) ,and a third, mandibular primordium defects (Table 1; Fig. 6D).

Morphological measurements of facial primordia of these embryos with defects on one side of the face, 48 hr after treatment, revealed quantitative differences between primordia on either side of the face. Normal untreated stage 22 embryos and sense ODN treated embryos had facial primordia that were equal in shape and size on both sides of the face (Figs. 6A–C, 7A–F). In most antisense-treated embryos, primordia on one side of the face were the same as controls while the opposing primordia were smaller. The antero-posterior extent of maxillary primordia was very significantly reduced and proximodistal outgrowth was affected in most cases (Figs. 6D–F, 7A,B). Nasal pits were shorter (proximodistally) and significantly wider (mediolaterally) (Figs. 6G,H, 7C,D). No statistically significant changes in growth of mandibular primordia could be detected (Fig. 7E,F). Embryos treated between stages 12 and 14 showed a similar incidence of defects (Table 1) and measurements showed similar quantitative differences to those seen in embryos treated between stages 10 and 11. With treatment between stages 15 and 18, only one out of five surviving embryos had facial defects, while treatment at stage 20–24 produced no defects in facial primordia (Table 1).

Other embryos treated with either sense, or antisense ODNs or pluronic gel, between stages 10–14, were incubated up to stage 34–5 to investigate later facial morphology. In all surviving embryos treated with connexin43 antisense ODNs (4/7), the thickened posterior maxillary primordium (tomium) was reduced or even absent (Fig. 8C–F; compare with controls in Fig. 8A,B). In 3/4 cases, this defect was unilateral and associated with lack of fusion between mandibular and maxillary primordia on the affected side resulting in a lateral facial cleft (macrostomia) (Fig. 8D–F). In the remaining case, both sides of the face were affected resulting in an excessively wide mouth (Fig. 8C). This contrasts with normal and control embryos at stage 34–5, in which the upper and lower beaks have a thickened edge/lip and are joined laterally and the developing upper beak, with egg tooth, overlaps the lower beak (Fig. 8A,B).

Effect Of Connexin43 Antisense ODNs on *Msx-1* Expression and Cell Proliferation in Facial Primordia

Patterns of connexin43 protein expression and *Msx-1* transcripts in face, limb, and neural tube are remark-

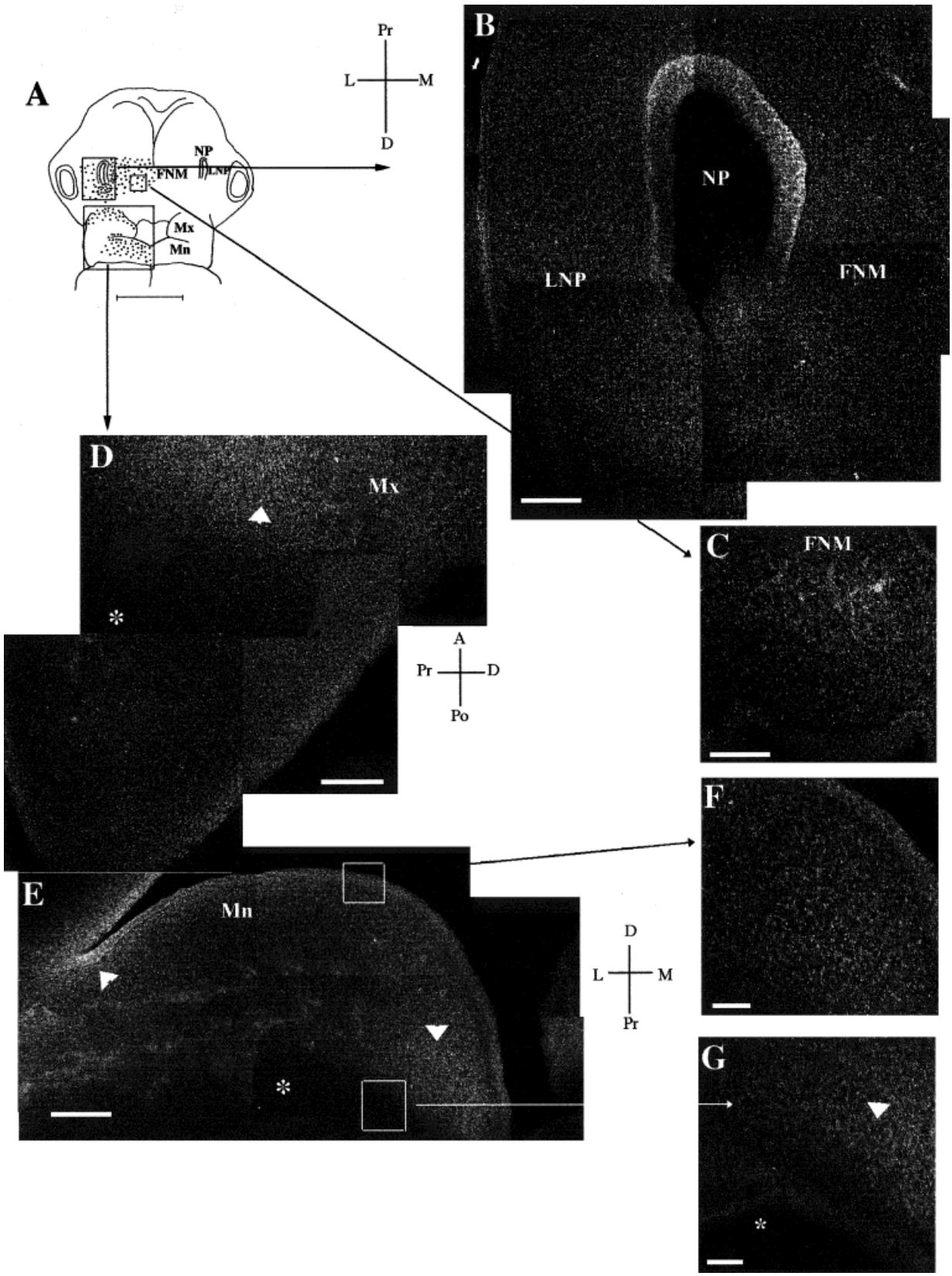


Fig. 4. Expression of connexin32 in stage 24 chick facial primordia. **A:** Diagram of chick head, stage 24. Black dots summarise regions of abundant connexin32 expression in the face as shown in B–G. Scale bar = 1,000 μm. **B:** Connexin32 protein abundant in superficial epithelia of nasal pit, and uniformly distributed in adjacent mesenchyme of lateral nasal process and frontonasal mass. **C:** Uniform connexin32 expression in central region of frontonasal mass. **D:** Connexin32 protein more abundant in anterior regions of maxillary primordium. (arrowhead), fewer gap junctions are seen proximally (*). **E:** Connexin32 protein distributed throughout mandibular primordium, particularly at proximal midline (arrowhead, right). Less expression is found proximally (*). Intense labelling can also be seen at merging point of mandibular and maxillary primordia (arrowhead top left). **F:** Connexin32 expression throughout distal edge of mandibular primordium. **G:** High levels of connexin32 expression close to proximal midline region of mandibular primordium (arrowhead), fewer connexin32 gap junctions are seen laterally (*). Scale bar = 100 μm in B–G. Figure and axes labels as in Figures 1 and 2.

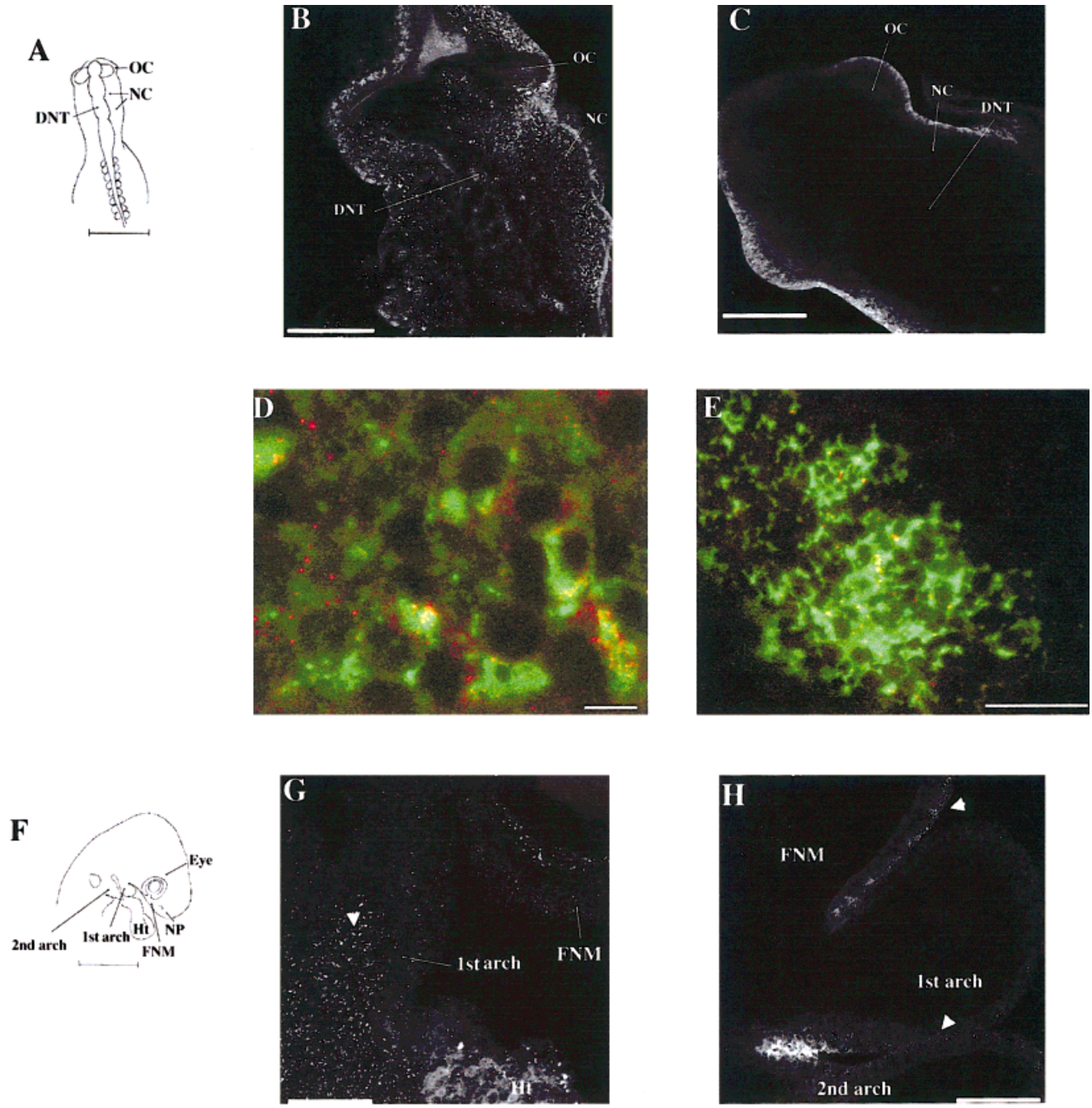


Fig. 5. Connexin43 expression in chick head and facial primordia after treatment with connexin43 antisense ODNs. **A:** Diagram of dorsal view of stage 11–12 chick embryo. Dorsal neural tube (DNT) runs along anterior-posterior axis of the embryo. Neural crest (NC) migrates laterally from edges of neural tube. Optic cups (OC). **B:** Connexin43 expression in dorsal neural tube (DNT) and tissue lateral to this, through which neural crest cells migrate, stage 11 chick embryo. **C:** Connexin43 expression in chick head, 8 hr after application of connexin43 antisense ODNs (stage 12). Little expression is seen in this embryo. **D:** Double immunolabelling of HNK-1, cell surface neural crest marker (green) and connexin43 (red) in midbrain neural crest (stage 10-). HNK-1 labelled cells have abundant connexin43 gap junction labelling. Areas of particularly high levels of HNK-1/connexin43 overlap appear yellow and coincide with borders between cells (scale bar = 10 μm). **E:** HNK-1 and connexin43 in the 1st pharyngeal arch stage 14. Neural crest is labelled with HNK-1 (green) and connexin43 (red). Non-crest mesenchyme surrounding neural crest was not labelled with either antibody (scale bar = 50 μm). **F:** Diagram of stage 17 chick embryo head (side view). Developing nasal pit (NP); Frontonasal mass (FNM); Heart (Ht). **G:** Connexin43 expression in chick face (side view, stage 17). Abundant connexin43 expression in 1st arch (arrowhead), frontonasal mass (FNM), and heart (Ht). **H:** Connexin43 expression in stage 17 1st arch primordia, 24 hr after connexin43 antisense application. Note little mesenchymal expression detectable in frontonasal mass and 1st arch primordium (arrowheads), but there is sparse epithelial staining. Note area of non-specific edge staining between 1st and 2nd arch.

I: Diagram of chick head, stage 22. Boxes indicate regions shown in J–M. Black dots summarise patterns of connexin43 expression after antisense treatment. **J:** Lateral nasal process, 48 hr after connexin43 antisense ODN treatment. Patches of connexin43 protein immunolabelling can be seen at lateral and distal edges (arrowheads), indicating return of connexin43 protein expression. **K:** Connexin43 expression in distal frontonasal mass. Again, some gap junctions can be seen in this tissue. Arrowhead indicates non-specific staining at base of nasal pit. **L:** Connexin43 expression in stage 22 maxillary primordium, 48 hr after application of connexin43 antisense ODNs. A few gap junction plaques are observed proximally (arrowhead), but generally, expression is still significantly reduced. **M:** Connexin43 expression can be seen in mesenchyme close to midline of mandibular primordium (arrowhead). This pattern is similar to that seen in untreated stage 22 mandibular primordium. Figure labels as before. Scale bars = 1,000 μm in A,F,I, 100 μm in B,C,G,H,J–M.

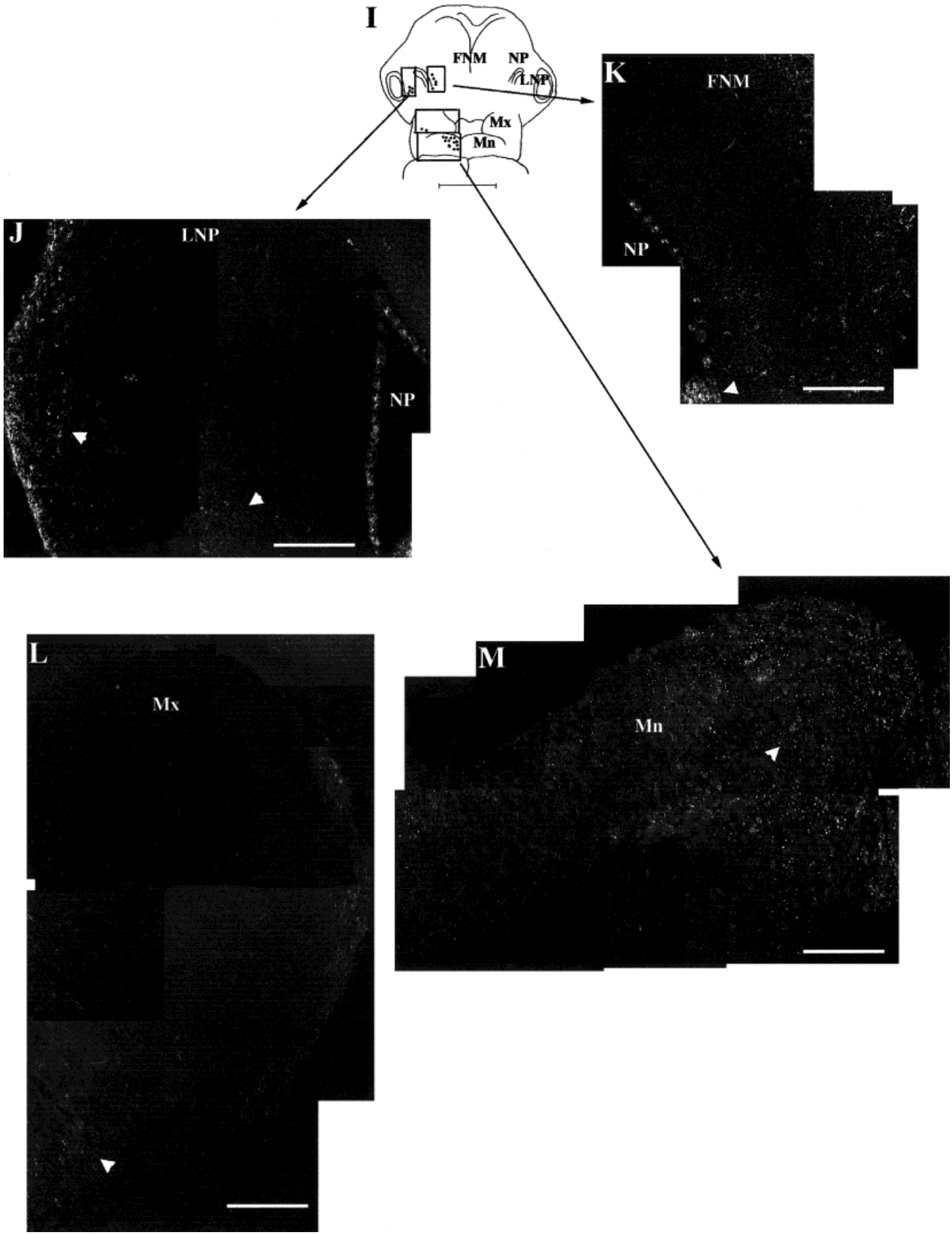


Figure 5 (Continued)

TABLE 1. Incidence of Facial Defects in Connexin43 Antisense or Sense ODN or Pluronic Gel Treated Chick Embryos

Stage of embryo treated	No. embryos with facial defects/no. surviving embryos (%)			Affected individual primordia after antisense treatment/no. embryos with facial defects (%)		
	Pluronic gel	Sense	Antisense	FNM/NP	Mx	Mn
10–11	0/12 (0)	0/7 (0)	9/19 (47)	4/9 (44)	9/9 (100)	3/9 (33)
12–14	0/10 (0)	0/17 (0)	10/17 (59)	5/10 (50)	10/10 (100)	3/10 (30)
15–18	0/4 (0)	0/2 (0)	1/5 (20)	1/1 (100)	1/1 (100)	1/1 (100)
20–24	0/5 (0)	0/7 (0)	0/5 (0)	0/0 (0)	0/0 (0)	0/0 (0)

ably similar (see Discussion). To test the relationship between connexin43 and *Msx-1*, connexin43 antisense ODNs were applied to chick embryos at stage 10. *Msx-1* expression was then monitored at various later time points. Changes were first detected at stage 17, 24 hr after treatment, when a reduction in *Msx-1* expression could be detected in medial regions of the first arch (Fig. 9C; compare with control embryos in Fig. 9A,B). Forty-eight hours after treatment (stage 22), levels of *Msx-1* expression were still reduced in maxillary and mandibular primordia and nasal pits of antisense treated embryos (Fig. 9F) compared to controls (Fig. 9D,E). Note that expression was also reduced in the neighbouring 2nd arch, which the antisense containing pluronic gel would also have covered.

Regions in which connexin43 protein is abundant are also those regions that we have previously shown to have high rates of cell proliferation (McGonnell et al., 1998). A statistically significant reduction in mitotic figures was observed in facial primordia of antisense treated embryos (Table 2), specifically in those regions that showed a transient reduction in growth upon antisense treatment, such as distal edge of the mandibular primordium (region b). A more highly significant reduction ($P < 0.001$) in mitotic figures was observed in regions of facial primordia involved in more permanent defects, namely posterior maxillary primordium bordering mandibular primordium (regions a and f in Table 2). It should be noted that there was a significant increase in proliferation in one region (c) of the mandibular primordia.

Expression of Connexin43 and 32 in Upper Beak Primordia After Retinoic Acid Treatment

Retinoic acid causes defects in the developing chick face when applied between stages 20 and 24. When retinoic acid was applied to the stage 20 chick face, expression of connexin43 protein was reduced in both epithelia and mesenchyme (n = 5 in each group). In distal nasal pit epithelia 12 hr after treatment, expression of connexin43 was reduced slightly (stage 22, Fig. 10B). By 24 hr, expression was significantly reduced (stage 24, Fig. 10C, D), and the nasal pits were noticeably shortened. Connexin43 expression was also reduced in the epithelium of distal anterior maxillary primordium (Fig. 10E). Connexin43 expression appeared to have returned to normal levels in all epithelia at 48 hr.

Mesenchymal expression of connexin43 was unchanged 12 hr after retinoic acid treatment (Fig. 10B). However, after 24 hr, connexin43 expression was reduced in mesenchyme of distal and lateral nasal pits (Fig. 10C, D), frontonasal mass and anterior distal maxillary primordia (Fig. 10E) and after 48 hr, was still reduced in mesenchyme of distal lateral nasal processes and anterior maxillary primordia. However, connexin43 distribution and abundance in frontonasal mass appeared normal, despite primary palate clefting.

In contrast to these effects on connexin43 expression, no change in connexin32 expression in either facial epithelia or mesenchyme could be detected at all time points examined (12, 24, 48 hr after treatment, n = 5 in each group, data not shown).

DISCUSSION

Expression of Connexin43 and 32 in Facial Primordia

Connexin43 protein varies in abundance in difference regions of the face. In contrast, mesenchymal and epithelial expression of connexin32 protein was much more uniform (see also Minkoff et al., 1991). This suggests that connexin43 and 32 play different roles in facial development.

Abundant connexin43 protein was found in mesenchyme in regions of the developing chick face that undergo considerable expansion and display high rates of cell proliferation (McGonnell et al., 1998). In other systems, the presence of connexin43 gap junctions has been both positively and negatively correlated with rates of cell proliferation depending on cell type (Becker and Mobbs 1999; Gibson et al., 1994, 1997; Zhu et al., 1991, 1992). The distribution of connexin43 gap junction protein in the face could explain why expansion and cell proliferation are confined to specific regions because, although there is no evidence of cell lineage compartments in the developing facial primordia at these stages (McGonnell et al., 1998), communication between different types of gap junctions may allow functional compartmentalisation.

Connexin43 protein was also abundant in mesenchyme and epithelia of regions where facial primordia attach and fuse. The significance of high levels of connexin43 in fusing primordia is unclear, but transient increases in the numbers of gap junctions have also been noted during primary palate fusion in the mouse (Kosaka et al., 1985).

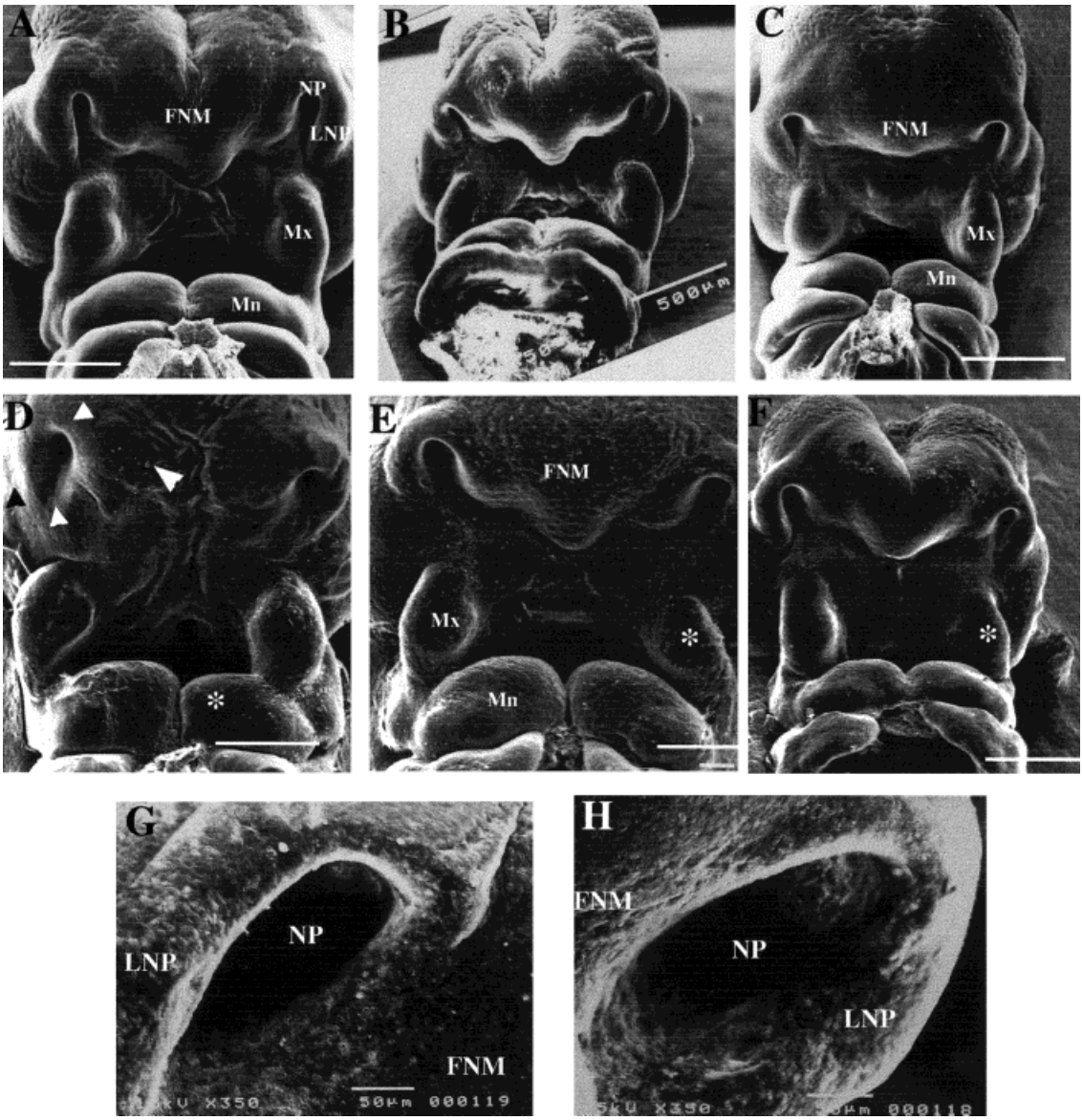


Fig. 6. SEM of chick facial primordia after treatment with connexin43 antisense ODNs. **A:** SEM of untreated stage 23 chick head. Primordia are equal in shape and size on either side of the face. **B:** SEM of chick head, stage 22, 48 hr after application of connexin43 sense ODNs. Primordia in these embryos are equal, similar to untreated controls. **C:** SEM of chick head, stage 22, 48 hr after treatment with pluronic gel. Primordia are again unaffected by this treatment. **D:** SEM of chick head stage 22, 48 hr after treatment with connexin43 antisense ODNs. Embryo is affected on right and left sides of the face. Left nasal pit is wider and misshapen, particularly at base (bottom white arrowhead). Left lateral nasal process is flattened (black arrowhead). Top of nasal pit is flattened (top white arrowhead), as is frontonasal mass (central white arrowhead). Right bud of paired mandibular primordium is shorter (*). Left hand maxillary primordia is short and does not contact mandibular primordia. **E:** SEM of stage 22 chick head 48 hr after treatment with connexin43 antisense ODNs. Right maxillary primordium (*) is smaller than contralateral control primordium in this embryo. **F:** SEM of stage 22 chick head 48 hours after treatment with connexin43 antisense ODNs. Right maxillary primordium (*) is smaller in this embryo. **G:** SEM of right nasal pit of a connexin43 antisense ODN treated embryo at stage 24. Lateral (LNP) edge of nasal pit is thickened and the pit tilts towards centre of the face. **H:** SEM of stage 24 left nasal pit (same embryo as in G), 48 hr after treatment with connexin43 antisense ODNs. Nasal pit (NP) is wider and tilted in the opposite direction compared to G. Surrounding tissue, particularly laterally (LNP), is also flatter. Scale bar = 500 μm in A–F. Scale bar = 50 μm in G and H. For abbreviations, see Figures 1–5.

Application of Connexin43 ODNs Results in Facial Defects

We used an antisense approach to test whether connexin43 has a causal role in expansion and fusion of chick facial primordia. Reduction of connexin43 expression throughout the developmental period between stages 12 and 22, or between stages 17 and 26, led to detectable changes in all facial primordia. The growth of the maxillary primordium was significantly reduced and this resulted in later fusion defects, namely lateral

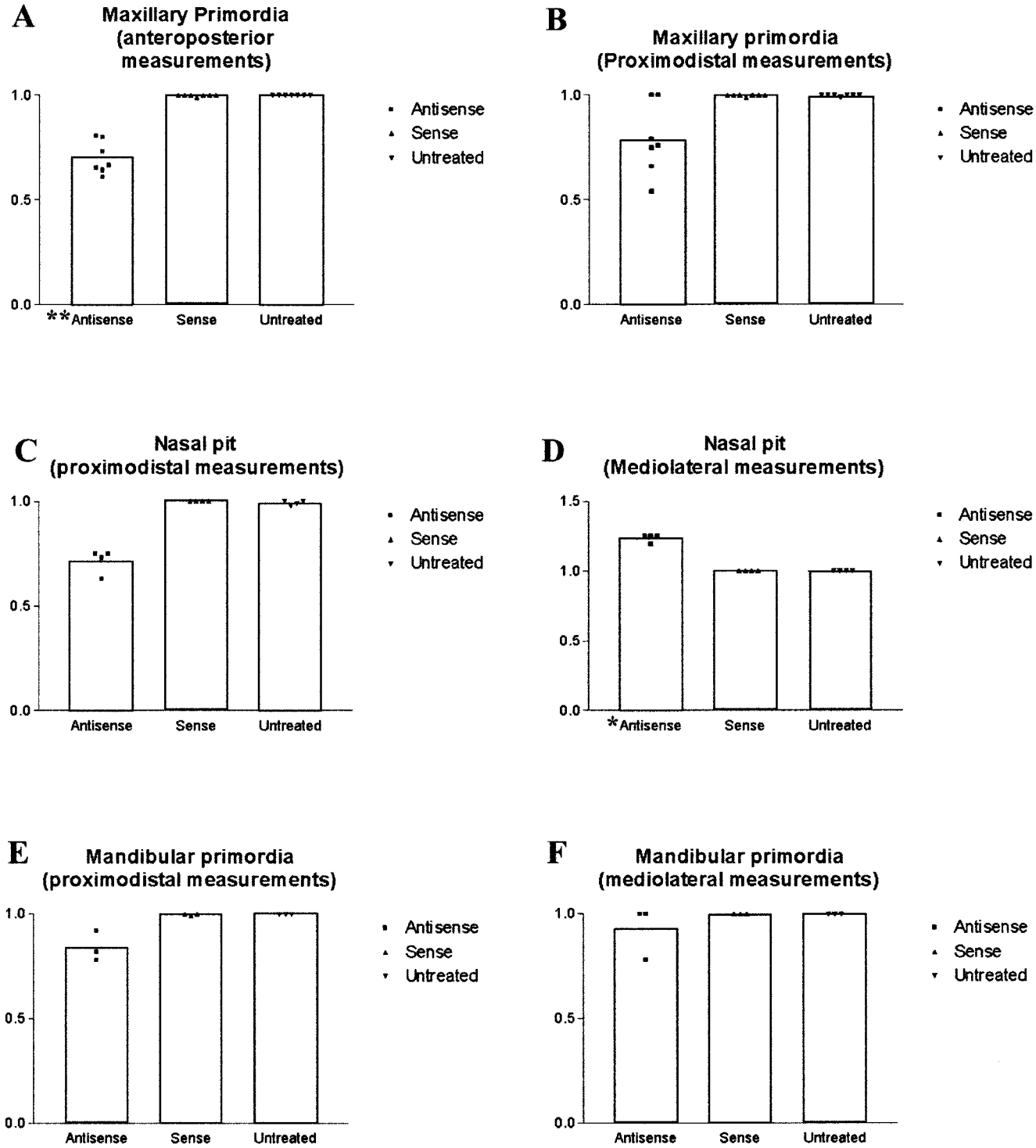


Fig. 7. Morphological measurements of facial primordia in Connexin43 antisense ODN treated embryos. Comparison with sense and untreated control embryos. **A:** Anteroposterior extent of maxillary primordia is very significantly reduced in antisense treated embryos. **B:** Proximodistal outgrowth of maxillary primordia is reduced in most antisense treated embryos. **C:** Proximodistal growth of the nasal pit is reduced in all antisense treated embryos but is not significantly different to controls. **D:** Mediolateral extent of the nasal pit is significantly wider in antisense treated embryos. **E:** Proximodistal outgrowth of mandibular primordia is not significantly reduced in antisense treated embryos. **F:** Mediolateral growth of mandibular primordia is also not significantly reduced. $^{**}P < 0.0001$; $^{*}P < 0.005$.

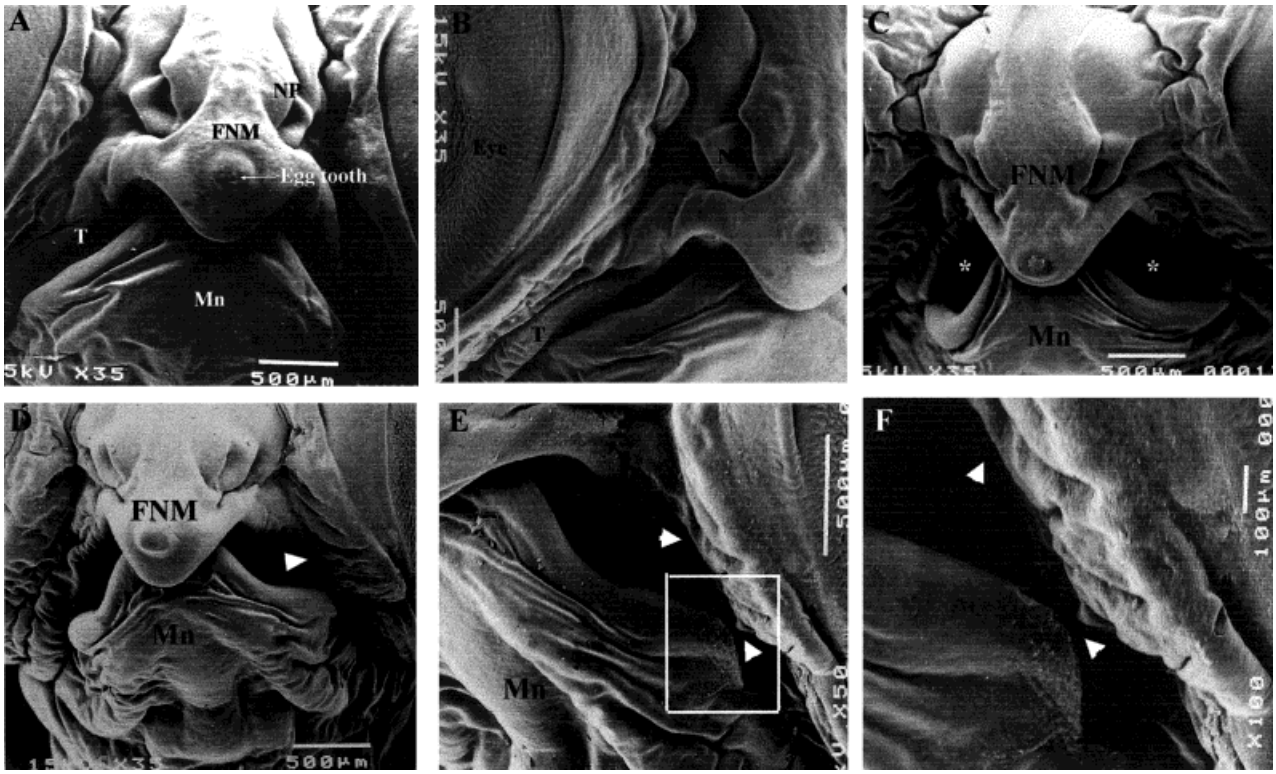


Fig. 8. SEM of control and antisense ODN treated stage 34 chick embryo heads. **A:** SEM of normal stage 34 chick embryo head. Upper beak, derived mainly from frontonasal mass, juts out over lower beak, derived from mandibular primordia. Edge of upper beak primordium, tomium (T), is derived from maxillary primordia. Egg tooth is epithelial thickening found only on upper beak. Nasal pits lie proximally on upper beak. **B:** Side view of normal stage 34 embryo. Tomium (T) of upper beak is more obvious from this view, as is the join between maxillary and mandibular primordia. **C:** SEM of stage 34 embryo, treated with connexin43 antisense ODNs at stage 10. Embryo affected on both sides of the face and has an excessively wide mouth (*). Tomium is reduced/missing on both sides of the face. **D:** SEM of stage 34 embryo treated with connexin43 antisense ODNs at stage 12. Embryo affected on left side of face. Mouth gapes open and tomium is much reduced on left (arrowhead). **E:** SEM of left side of embryo in D. Tomium is reduced/missing (top arrowhead). There is failure of fusion between maxillary and mandibular primordia (bottom arrowhead). Box indicates region at higher magnification in F. **F:** Tomium is missing (top arrowhead) and there is a wide gap between mandibular and maxillary primordium (bottom arrowhead). Scale bars = 500 μm in A-E, 100 μm in F.

facial clefts (macrostomia). The growth of other primordia was also reduced at early stages but this had no lasting effects on primary palate formation and beak outgrowth. This could be due to the fact that reduction of connexin43 expression in frontonasal mass was more transient than in maxillary primordium.

Both early and late facial defects were generally unilateral. Chick embryos turn to lie on one side, usually the right, at stage 13. Thus, only one side of the face is exposed to antisense ODNs for most of the treatment period and we expected most defects to be on the right hand side of the face. However, 40% of treated embryos turned to the left, and in these embryos the facial defects were on the left. This was the initial indicator of which side of the face was affected and was recorded for each embryo upon opening of the egg. This randomisation of turning direction may be due to the weight of pluronic gel, as direction of turning is known to be specified prior to the earliest stage of treatment (Levin et al., 1995) and, interestingly, involves communication through connexin43 gap junctions (Levin and Mercola, 1998, 1999).

The chick facial defects found here are similar to those recently observed in connexinx43 null mice (Lecanda et al., 2000). In these mutant mice, both mandibular and maxillary primordia were shorter than normal. Shortening of the maxillary primordia mirrors the reduction in maxillary primordia outgrowth seen in antisense treated chick embryos. Functional compensation by other connexins may explain why other defects seen in the chick embryos, such as clefting, were not observed in the connexin43 null mice. Indeed, Lecanda et al. (2000) noted upregulation of connexin45 expression in these mice.

Antisense ODNs against mRNA for other molecules thought to be involved in facilitating gap junction formation have been applied to both chick and mouse embryos and these produce remarkably similar facial phenotypes to those produced by connexin43 antisense ODNs. Thus, antisense to mRNA for the cell adhesion molecule E-cadherin (Chen and Hale, 1995) and for signalling molecules implicated in cell coupling, Wnt-1 and Wnt-3a (Augustine et al., 1993), all resulted in hypoplasia of facial primordia. These results are fur-

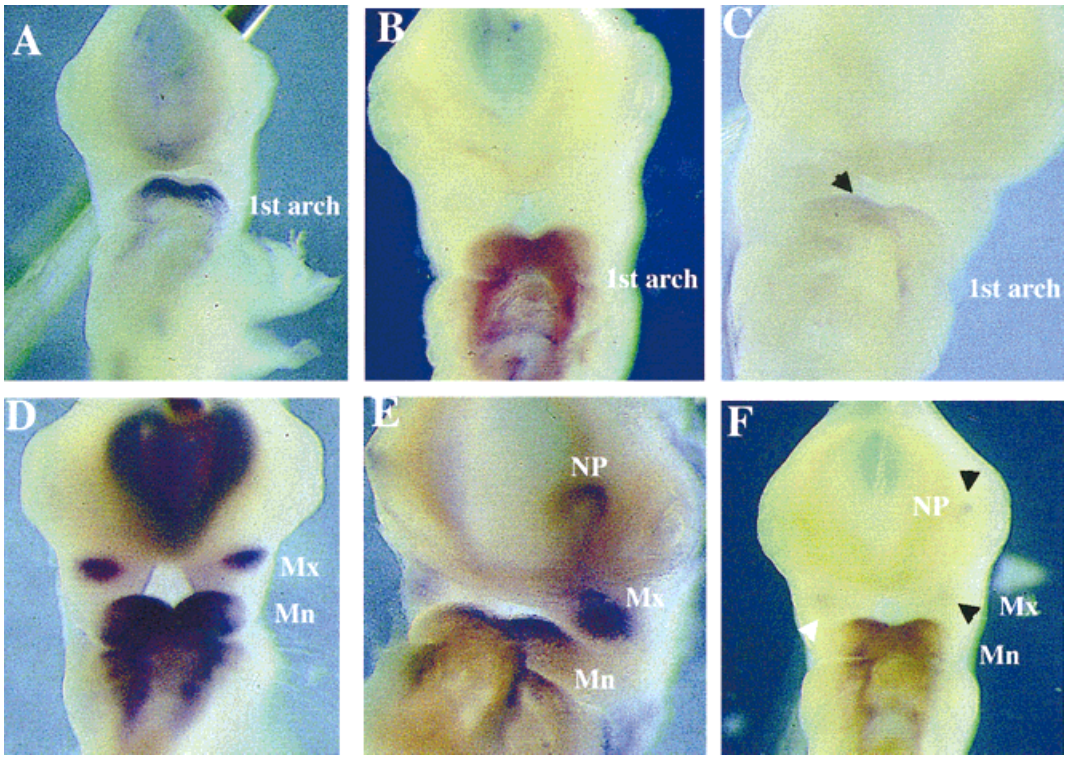


Fig. 9. Expression of *Msx-1* transcripts in chick head and facial primordia after application of connexin43 antisense ODNs. **A:** Stage 16/17 embryo, 24 hr after treatment with pluronic gel (control). *Msx-1* transcripts throughout first arch primordium (1st arch). Note slight trapping of colour reagent in the forebrain, which does not represent Msx-1 expression. **B:** Stage 17 embryo, 24 hr after treatment with connexin43 sense ODNs (control). As in A, *Msx-1* expression in 1st arch primordium. **C:** Stage 17 embryo, 24 hr after treatment with connexin43 antisense ODNs. Low levels of *Msx-1* transcripts in distal 1st arch primordium (arrowhead). **D:** Stage 22 embryo, 48 hr after application of pluronic gel (control). High levels of *Msx-1* transcripts in anterior maxillary (Mx) and in mandibular (Mn) primordia. There is significant trapping of colour reagent in the forebrain neural tube in this embryo. **E:** Stage 24 embryo, 48 hr after application of connexin43 sense ODNs (control). *Msx-1* transcripts in nasal pit, anterior maxillary primordium, and mandibular primordium. Embryo is at an angle so that proximal mandibular primordium is slightly obscured. **F:** Stage 23 embryo, 48 hr after treatment with connexin43 antisense ODNs. *Msx-1* transcripts are expressed at lower levels than normal in mandibular primordium, compare with D,E. Low levels of *Msx-1* expression in both maxillary primordia (white arrowhead on the left, black arrowhead on right) and in right nasal pit, (top black arrowhead) but are absent in left nasal pit. For abbreviations, see Figures 1–5.

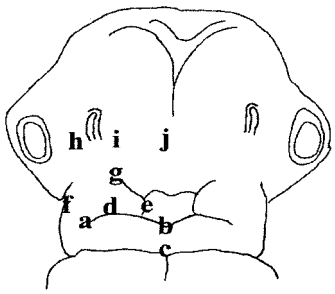
ther confirmation that cell-cell communication via connexin43 containing gap junctions is important for correct facial development.

Retinoic Acid Reduces Expression of Connexin43 Protein in Chick Facial Primordia

Defects in expansion and fusion of facial primordia are produced by retinoic acid. Therefore, we examined whether gap junction protein expression was perturbed by retinoic acid treatment of the face. We found that retinoic acid reduces connexin43 but not connexin32 expression. Retinoic acid treatment has previously been reported to reduce connexin43 expression and gap junction coupling in Syrian hamster embryo cells and neuronal cells in vitro (Bani-Yagoub et al., 1997; Rivedal et al., 1994) and connexin43 expression in ectoderm and mesenchyme of developing chick limb buds in vivo (Green et al., 1994). In contrast, retinoic acid increased connexin43 expression and intercellular communication in F9 teratocarcinoma cells in vitro (Clairmont et al., 1996).

In the chick face, regions in which connexin43 protein expression was decreased by retinoic acid are those that contribute to primary palate formation namely, distal lateral nasal processes, bases of nasal pits, and distal maxillary primordia. The fact that connexin43 expression in these regions was reduced following retinoid treatment and that connexin43 antisense also inhibits expansion and cell proliferation suggests that retinoid defects could arise via reduced gap junctional communication. If this is the case, why are facial defects induced by retinoids (primary palate clefting) different from those produced by connexin43 antisense ODNs (macrostomia)? In hamsters, retinoid treatment on E8 resulted in reduced growth of maxillary primordia and macrostomia without primary palate clefting whilst treatment on E9 resulted in primary palate clefting only (Irving et al., 1986). Therefore, the fact that our antisense treatments reduce connexin43 expression at earlier stages in chick facial development than our retinoid treatments may account for the differences in outcome.

TABLE 2. Mitotic Indices in Regions of Facial Primordia After Treatment With Sense and Antisense ODNs



Primordium	Average no. mitotic figures per standard unit area (Control)	Treated
Mandibular primordia		
a	5.4 (n = 7)	0.5 (n = 8)**
b	7.6 (n = 5)	4.3 (n = 7)*
c	5.5 (n = 6)	7.5 (n = 8)*
Maxillary primordia		
d	3.0 (n = 5)	1.0 (n = 11)*
e	2.4 (n = 5)	1.8 (n = 10)
f	4.6 (n = 5)	2.0 (n = 11)**
g	4.4 (n = 5)	3.5 (n = 10)
Frontonasal mass/lateral nasal process		
h	4.8 (n = 5)	1.5 (n = 10)**
i	3.4 (n = 5)	2.2 (n = 9)*
j	1.6 (n = 5)	1.1 (n = 8)

*$P < 0.01$.
**$P < 0.001$.

Connexin43 and Msx-1 Are Implicated in Outgrowth of Facial Primordia

We found that connexin43 antisense treatment reduced levels of *Msx-1* mRNA in facial primordia, suggesting that gap junctional communication is necessary for expression of *Msx-1* transcripts. Spatiotemporal expression patterns of connexin43 protein and *Msx-1* transcripts are strikingly similar in developing face (Brown et al., 1993; Minkoff et al., 1997) neural tube (Ruangvoravat and Lo, 1992; Suzuki et al., 1991) and limb (Davidson et al., 1991; Green et al., 1994). Furthermore, retinoic acid treatment of chick embryos not only reduces connexin43 expression, as we have shown here, but also results in decreased *Msx-1* expression in facial primordia (Brown et al., 1997). *Msx-1* null mice have shorter mandibular primordia and clefting of the secondary palate (Satokata and Maas 1994), and when *Msx-1* antisense ODNs were applied to cultured mouse embryos, similar defects to those observed here with connexin43 antisense were obtained (Foerst-Potts and Sadler, 1997). Thus, we hypothesise that connexin43 and *Msx-1* are required, perhaps even in the same pathway, to control outgrowth and fusion of facial primordia.

Connexin43 May Play a Role in Several Phases of Facial Development

Connexin43 protein is found in chick cranial neural crest cells during several stages of facial development, from migration to later outgrowth of facial primordia. Connexin43 expression and functional coupling has been reported in migrating cranial and trunk neural crest of mouse embryos (Lo et al., 1997) and it has been proposed that alterations in neural crest migration underlie the heart malformations seen in mice when *connexin43* expression is manipulated (Ewart et al., 1997, Huang et al, 1998). More recently, defects in ossification of cranial bones in connexin43 null mice have been partially attributed to reduced numbers of neural crest cells. Disrupted cell-cell signalling between migrating neural crest cells in our connexin43 antisense treated embryos may contribute to reduction in size of facial primordia. Indeed, treatment of early avian and mammalian embryos with retinoic acid reduces numbers of migrating neural crest cells resulting in similar facial defects (Pratt et al., 1987; Thorogood et al., 1982). However, connexin43 antisense also led to defects in embryos treated after stage 12. In these embryos, connexin43 protein levels would not have been reduced until after cranial neural crest migration was complete (stage 13/14), supporting the idea that junctional communication mediated by connexin43 is also important at later stages in face development, once primordia have been filled by neural crest cells. We also showed directly that, in antisense-treated embryos, cell proliferation was significantly reduced in areas of facial primordia involved in expansion and fusion. These are areas in which connexin43 containing gap junctions are normally very abundant. The most significant reduction in proliferation was found at the border of maxillary and mandibular primordia, the region in which the most persistent defect (macrostomia) was found.

METHODS

Embryos

Chick embryos were incubated at 38°. Eggs were windowed and staged according to Hamburger and Hamilton (1951). Embryos at appropriate stages were treated, in ovo, with either sense or antisense ODNs or retinoic acid. Eggs were re-incubated for various times and embryos removed for immunohistochemistry or in situ hybridisation experiments.

Immunohistochemistry

Single antibody labelling. Embryos were processed for immunohistochemistry as described in Becker et al. (1995). Treated embryos were fixed in 4% paraformaldehyde at various time points after manipulations and normal embryos were fixed at various stages. The heads of embryos were trimmed and blocked overnight in PBS/ 0.05% Triton X-100/ 0.1M lysine. They were subsequently incubated with connexin specific antibodies for up to 1 week at 4°C. This

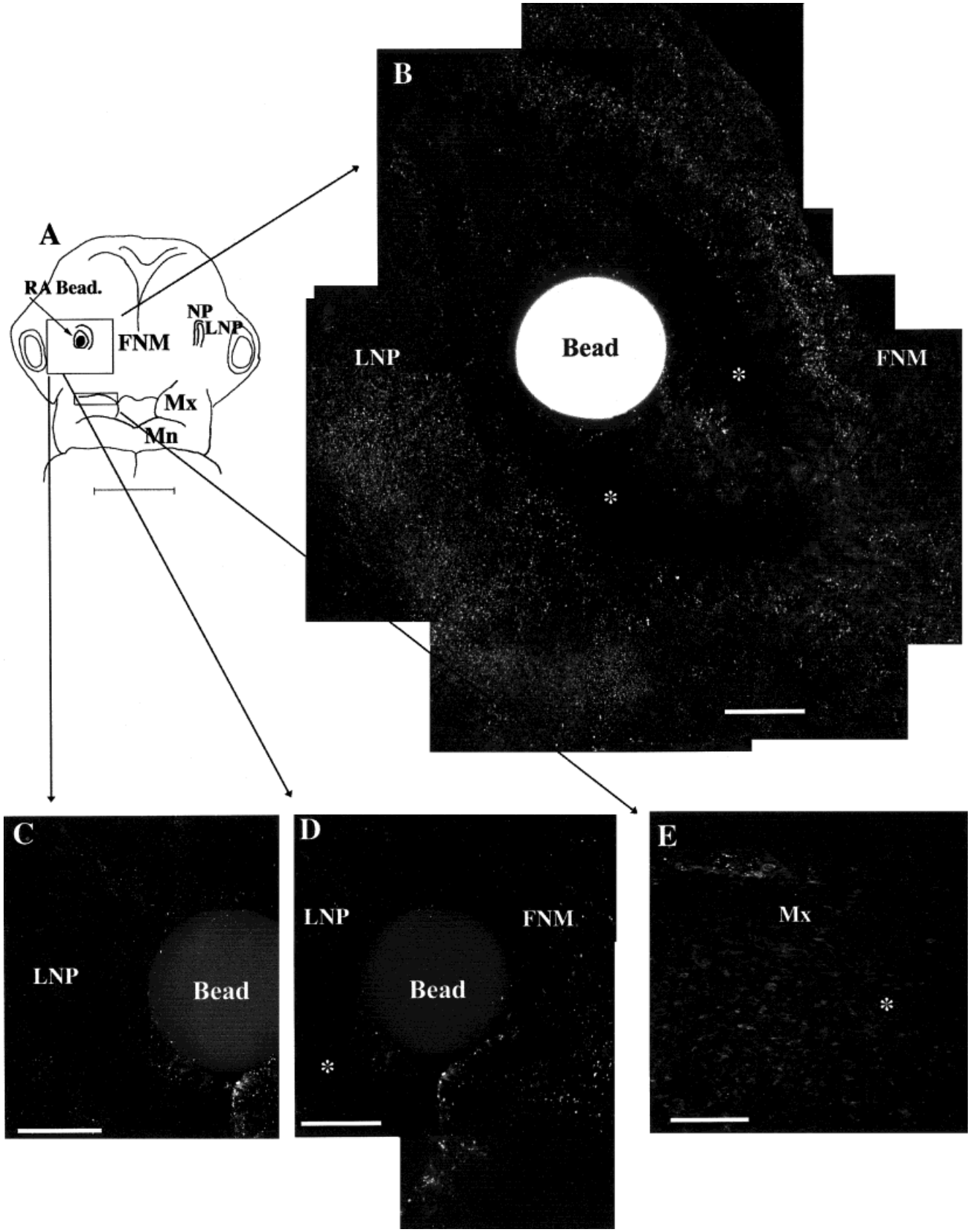


Fig. 10. Expression of connexin43 in chick upper beak primordia after treatment with retinoic acid. **A:** Diagram of chick embryo head, stage 24. Retinoic acid soaked bead is in right nasal pit. Right nasal pit and maxillary primordium show defects characteristic of retinoic acid treatment. Scale bar = 1,000 μm. **B:** Connexin43 expression reduced in surface epithelia of nasal pit (*), 12 hr after treatment. **C:** Connexin43 expression reduced in lateral nasal process 24 hours post retinoic acid treatment. **D:** Connexin43 expression reduced in frontonasal mass and almost abolished in lateral nasal process (*) 24 hr after treatment. **E:** Connexin43 in maxillary primordium 24 hr post retinoic acid treatment. A small patch of epithelial expression can be seen at the top of the picture. There is little mesenchymal expression (*). Scale bar (B–E) = 100 μm. For abbreviations, see Figures 1–5.

extended incubation period ensured total penetration of antibody. Polyclonal primary antibodies used were Gap15, specific for connexin43 (Becker et al., 1995; Gourdie et al., 1990) and Des5, specific for connexin32 (Becker et al., 1995, 1999b). Embryo heads were incubated with secondary antibody (swine anti-rabbit FITC) overnight, at 4°C, then mounted on cavity slides using Citifluor and examined at ×63 magnification at comparable depths on a Lieca TCS4D confocal microscope. At this magnification, each fluorescent spot indicates a gap junction plaque (see Green et al., 1994). Images were stored digitally for analysis. The z-range of this microscope is 80–90 μm.

Double antibody labelling. Stage 10–14 embryos were fixed, blocked, and labelled with primary connexin43 antibody as above. Embryos were then incubated in swine anti-rabbit TRITC overnight at 4°C, washed in PBS, and incubated with mouse anti-human HNK-1 (CD57) directly coupled to FITC (Pharmigen) overnight at 4°C. Embryos were washed and examined as above.

Construction of figures. Each single image in a z-series captures the distribution of connexin protein in a 0.5 μm section of the tissue (Fig. 1A). The z-series was then projected to give a single image showing the distribution of connexin protein in a tissue depth of up to 80–90 μm (Fig. 1B, see Becker et al., 1995, for methods). This provides a 2-dimensional summation of the number and distribution of antibody labelled connexin containing gap junctions in that volume of tissue. Overlapping z-series were then assembled into montages to show whole primordia using Adobe Photoshop 4.0. This allowed visualisation of the abundance and spatial pattern of connexin43 or 32 gap junctions in whole primordia. The overall size of each z-series image was reduced considerably (Fig. 1C) to make the montages, each containing at least 16 separate projected images, shown in Figure 3.

Antisense application. Eggs were windowed and membranes over the head were opened. Thirty percent Pluronic F-127 gel (BASF Corp) in PBS delivered unmodified connexin43 specific antisense ODNs to stage 10–24 developing chick embryos (Becker et al., 1999a, b). The gel is a mild surfactant expediting ODN penetration into cells and was placed over the region of the embryo from the anterior tip of the head to the heart. All ODNs were prepared from concentrated stock solutions, stored at −80°C, and applied at 0.5–1.0 μM final concentration in pluronic gel. Sense ODNs and pluronic gel alone were applied as controls. Details of antisense, sense, and control ODN characterisation appears in Becker et al. (1999a).

After antisense ODN application, eggs were reincubated for 2–48 hr or up to 8–9 days of development. Immunohistochemical localisation of connexin43 gap junction protein provided a direct measure of the antisense effect.

In situ hybridisation. In situ hybridisation was performed according to Nieto et al. (1996). Antisense Msx-1 riboprobe was transcribed from BamHI linearised template using T7 (Brown et al., 1993). Treated embryos were processed in the same tubes as untreated (control) embryos to ensure that full development of signal had occurred.

Cell proliferation. To determine effects of connexin43 antisense treatment on cell proliferation, embryos were treated at stage 10 with either antisense or sense ODNs and then incubated for 48 hr (stage 22, n = 5 for each treatment group), fixed in 4% paraformaldehyde, and embedded in 4% agar. Embryos were randomised and assigned a unique identifying number. Saggital sections (100 μm), cut on a vibroslice, were treated with 0.1 μm propidium iodide for 5 min, washed in PBS, and mounted in citifluor. Sections were examined on a confocal microscope. Mitotic figures are clearly visible using this technique (Becker and Mobbs, 1999). Counts of dividing cells were made in a random single focal plane in facial primordia. Data were then reassigned to either the treated or control groups and a One-tailed Mann-Whitney U-test, a non-parametric statistical test, was used to compare mitotic indices between treatment groups.

SEM and measurements. Embryos were treated with either antisense ODNs or controls and incubated up to 48 hr (up to stage 22 for embryos treated at stages 10/11). Development of the face was examined by light microscopy and primordia of embryos treated at stages 10–11 were also measured using a micrometer and graticule eyepiece. Data were analysed as the ratio of untreated:treated side of the face and plotted on a graph using Prism software (Graphpad Inc.). Measurements of treated and control data were compared statistically using the Krusskal-Wallis test, a non-parametric statistical test that does not presume that the data is normally distributed and also allows for small n numbers by taking them into account when producing the *P* value. Embryo heads were then fixed in modified Tyrodes solution, dried in a critical point dryer, sputter coated with gold, and examined in a Jeol scanning electron microscope.

Retinoic acid. Retinoic acid stock solution, 10 mg/ml in DMSO, was prepared from All-trans retinoic acid (Sigma, St. Louis, MO). AG1-X2 formate beads (100 μm diameter) were soaked in 10 mg/ml retinoic acid solution as in Eichele et al. (1984). Beads were placed into the right nasal pit of stage 20 embryos in ovo and incubated for 12, 24, or 48 hr.

ACKNOWLEDGMENTS

The authors thank Franklin Harris for initial work that led to these investigations, Jeremy Cook for help with statistics, and S. Wedden for *Msx-1 riboprobe*. I.M.M. was the recipient of a Glaxo-Wellcome studentship. C.R.G. thanks the Marsden Foundation for their support. C.T. and D.L.B. are funded by the MRC and D.L.B. is a Royal Society University Research Fellow.

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