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Original Article

Inhibition of TGF-ß$_3$ (but not TGF-ß$_1$ or TGF-ß$_2$) activity prevents normal mouse embryonic palate fusion

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ABSTRACT The critical stage of palatal development is the fusion of apposing individual palatal shelves. Palatal shelves, from the day 13 murine embryo, cultured *in vitro* fuse normally in the absence of exogenous factors. Therefore, some endogenous factor(s) is responsible for the normal fusion process. Prevention of mature TGF-ß$_3$ activity during a specific time window of development in palate organ cultures, either by antisense oligodeoxynucleotides or neutralizing antibody, resulted in failure of palate fusion. Northern analysis was used to demonstrate that the antisense treatment down-regulated TGF-ß$_3$ mRNA. Inhibition of TGF-ß$_1$ or -ß$_2$ activity (by either antibodies or antisense oligodeoxynucleotides) had no such effect on palate development and palate fusion was normal. These data indicate an isoform specific role for TGF-ß$_3$ in palatal fusion.

KEY WORDS: *TGF-ß, palate development*

Introduction

Palatogenesis in the mammalian embryo involves a complex series of events such as morphological movements, mesenchymal-epithelial interactions, cell differentiation, migration and transformation (Ferguson, 1988). Soluble growth factors, extracellular matrix molecules and their receptors play an interactive role during these processes (Sharpe and Ferguson, 1988). Perhaps the most critical stage in mammalian palate development is the formation of a mid-line seam between medial edge epithelial (MEE) cells of apposing palatal shelves. This occurs at mouse embryonic day 14.5 (E14.5) *in vivo* (Fig. 1A-B). Subsequent disruption of this epithelial seam leads to mesenchymal continuity and an intact, fused palate by E15 (Fig. 1C-D). Many investigations have sought to identify the growth factors present at the time of palatal fusion and the effect they have on palatal tissues. The expression of many growth factors in the region of the medial edge suggests that they may play an important role in palatal fusion. For example, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α; Abbott and Birnbaum, 1990; Dixon *et al.*, 1991), transforming growth factor-ß (TGF-ß; Heine *et al.*, 1987; Gehris *et al.*, 1991), fibroblast growth factor-1 and -2 (Sharpe *et al.*, 1993), insulin-like growth factor II (Ferguson *et al.*, 1992) and platelet-derived growth factor-A (Qiu and Ferguson, 1995) are all present in the medial edge epithelial seam, or underlying mesenchyme, at the time of palatal fusion. However, evidence for a physiological function of any one of these growth factors has been largely circumstantial, relying on immunocytochemical and *in situ* hybridization data which merely prove that a given factor is expressed at the right time and in the right place. Further clues to the developmental roles of palatal growth factors came from *in vitro* organ culture experiments where a given growth factor was added to palatal tissues and its effect on subsequent development observed. This approach revealed that exogenous TGF-ß$_1$, -ß$_2$ or -ß$_3$ accelerated palatal fusion (Dixon and Ferguson, 1992; Gehris and Greene, 1992; Brunet *et al.*, 1993), exogenous EGF (or TGF-α) blocked fusion (Hassell, 1975; Hassell and Pratt, 1977; Tyler and Pratt, 1980; Brunet *et al.*, 1993), while many growth factors altered the extracellular matrix composition of cultured palatal tissue (Silver *et al.*, 1984; Foreman *et al.*, 1991). While such observations are interesting, they may represent pharmacological actions of exogenous growth factors rather than physiological functions of growth factors endogenous to the developing palate.

Significantly, during *in vitro* palatal culture in serum-free, chemically-defined culture medium the apposing palatal shelves will fuse to form a mid-line epithelial seam which then disperses as *in vivo* (Ferguson *et al.*, 1984). Therefore, the factors necessary for normal mammalian palatal fusion are present within, or synthesised by, the embryonic palatal shelf.

We have therefore performed a series of *in vitro* experiments in which we have sought to neutralize growth factors endogenous to the embryonic murine palatal shelf using either specific antisense oligodeoxynucleotides (ODN) to prevent mRNA expression or neutralizing antibodies to block the activity of the active peptide.

The TGF-ß family was chosen since *in situ* hybridization and immunocytochemistry (Heine *et al.*, 1987; Fitzpatrick *et al.*, 1990;

Abbreviations used in this paper: EGF, epidermal growth factor; MEE, medial edge epithelia; ODN, oligodeoxynucleotide; TGF-α, transforming growth factor-alpha; TGF-ß, transforming growth factor-beta

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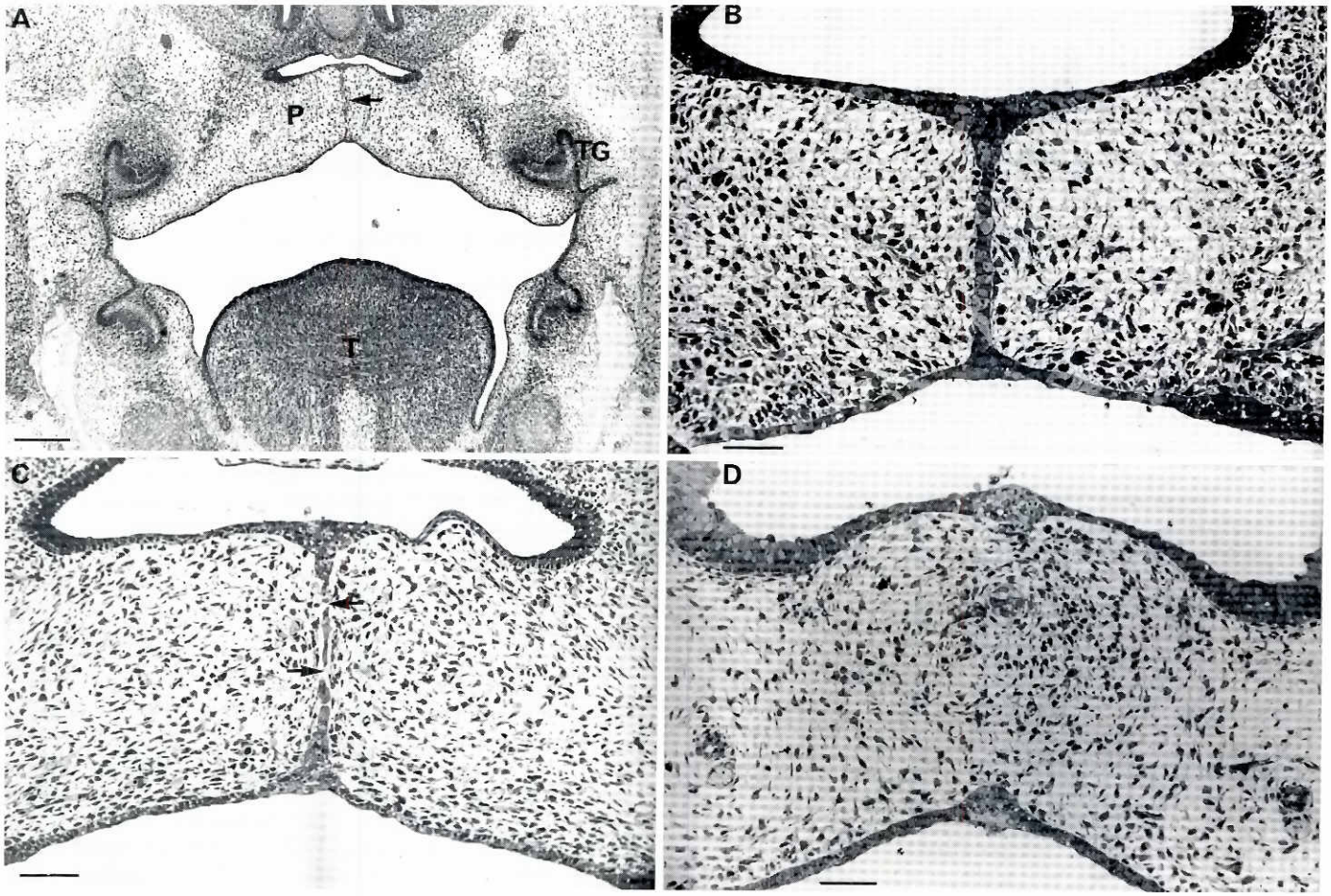

Fig. 1. Normal palate development. (A) *Photomicrograph of an E14.5 mouse palate in vivo. The MEE of apposing palatal shelves have adhered to one another and a mid-line seam has formed (arrowed) (P, palatal shelf; TG, tooth germ; T, tongue). Scale bar, 150 μm.* **(B)** *Higher power view of a similar stage palate as in* **(A)**. *Scale bar, 40 μm.* **(C)** *Later on day 14 the mid-line seam has begun to degenerate (arrowed). Scale bar, 40 μm.* **(D)** *By E15 this seam has completely dispersed, resulting in mesenchymal continuity across the palate. Scale bar, 40 μm.*

Pelton *et al.*, 1990; Gehris *et al.*, 1991) revealed that the three main TGF-ß isoforms (TGF-$ß_1$, $ß_2$, $ß_3$) are expressed in a temporally- and spatially-regulated manner throughout palatogenesis. In general, the expression patterns were non-overlapping suggesting that each isoform may have a specific function, a hypothesis we were able to test using isoform-specific deletive approaches. Such a deletion strategy has previously demonstrated that ODN neutralization of TGF-$ß_3$, but not TGF-$ß_1$ or TGF-$ß_2$, prevented the epithelial-mesenchymal transformation which occurs during development of the chick heart (Potts *et al.*, 1991; Runyan *et al.*, 1992).

Results

Treatment of palate organ cultures with antisense oligodeoxynucleotides

Treatment of paired palate organ cultures with antisense ODN to TGF-$ß_3$ at 10 μM throughout the culture period prevented normal palate fusion (Figs. 2C-D, 3C-D). By contrast, in control cultures by 48 h (Fig. 2A-B) the two palatal shelves adhered to each other and their MEE fused to form a midline epithelial seam similar to that which forms *in vivo* at embryonic day 14.5 (Fig. 1A-B). Antisense TGF-$ß_3$ treated cultures showed no signs of adherence at their medial edges and no seam had formed (Fig. 2C-D). The space between the palatal shelves is a fixation shrinkage artefact and occurred because the shelves had not adhered to each other when the tissue was submerged in the fixative. The two shelves were in contact throughout the entire period of organ culture.

By 72 h of culture, control tissue had undergone complete fusion, corresponding to the stage at embryonic day 15 *in vivo*. The mesenchyme is continuous across the explant and the MEE had completely disappeared (Fig. 3A-B). Antisense TGF-ß3 treated cultures had not fused by 72 h (Fig. 3C-D). Indeed, several layers of MEE could still be seen, indicating that normal seam formation had not occurred, although clearly the MEE had adhered. It is possible that the antisense oligodeoxynucleotide is delaying fusion rather than preventing it. Ideally we would like to extend the culture period for a further 24 h to determine this. However, this culture system is unsuitable for such analysis since tissue necrosis begins after 72 h, especially in the epithelia and it is well known that «sick» epithelia can fuse. Treatment with lower doses of antisense TGF-ß3 had no effect on palate development and

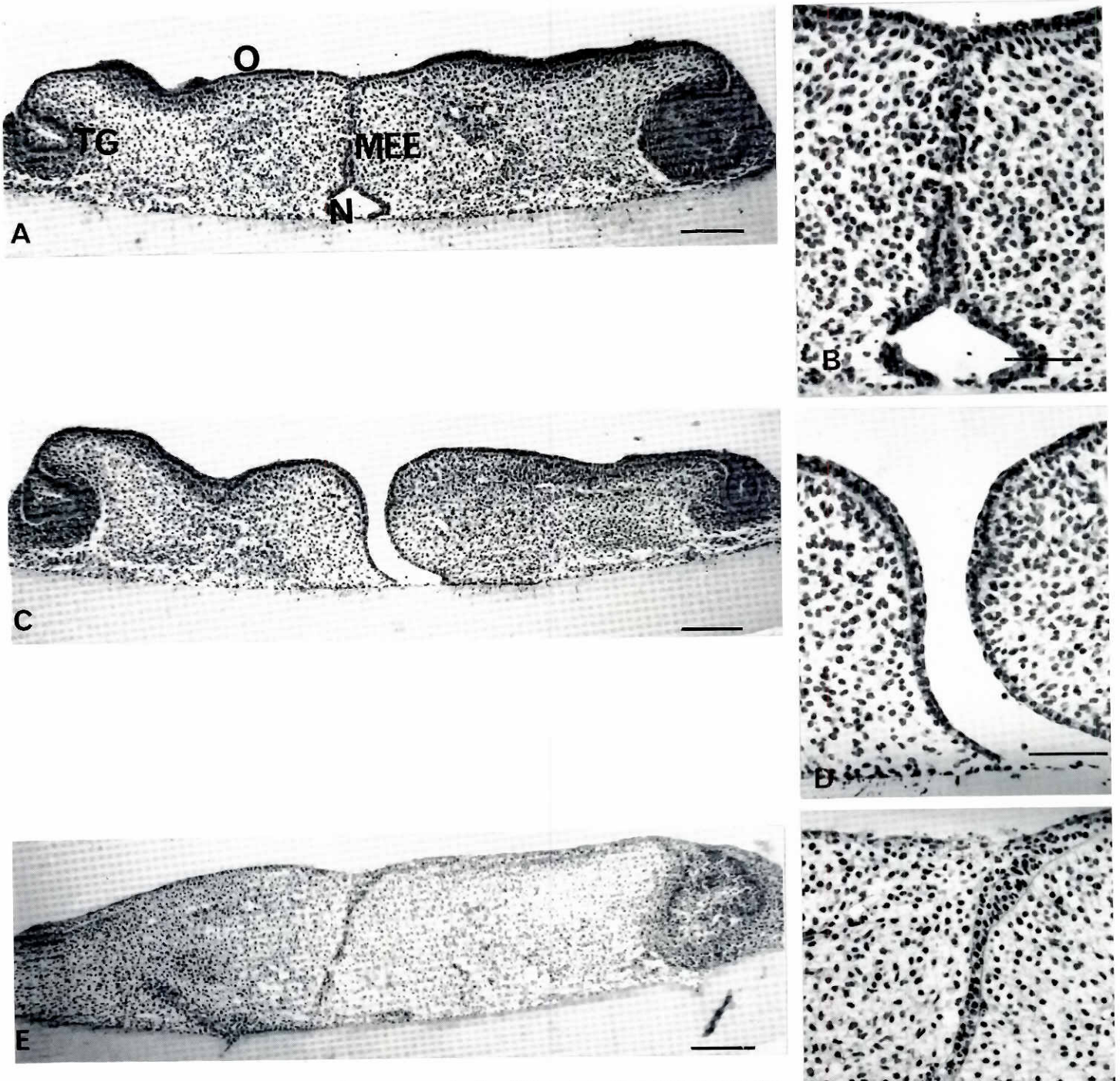

Fig. 2. Antisense treated cultures. (A) *Photomicrograph of an E13 control culture fixed at 48 h. The medial edge epithelia of the two palatal shelves fused to form a seam (MEE). The developing tooth germ (TG) is also indicated, as are the oral (O) and nasal (N) palatal epithelia. Scale bar, 50 μm.* **(B)** *Higher power magnification of the seam region in* **(A)**. *Scale bar, 100 μm.* **(C)** *Photomicrograph of an E13 culture incubated for 48 h in the presence of 10 μM TGF-ß$_3$ antisense ODN. The MEE of the two palatal shelves did not adhere and no seam formed. The space between the two shelves is a shrinkage artefact which occurred on fixation. Scale bar, 50 μm.* **(D)** *High power magnification of the non-adhering MEE in* **(C)**. *Scale bar, 100 μm.* **(E)** *Photomicrograph of an E13 culture incubated for 48 h in the presence of 10 μM TGF-ß$_3$ sense ODN. The MEE have approximated and formed a midline seam. Note similar appearance to untreated, control cultures* **(A-B)**. *Scale bar, 50 μm.* **(F)** *Higher power magnification of the seam region in* **(E)**. *Scale bar, 100 μm.*

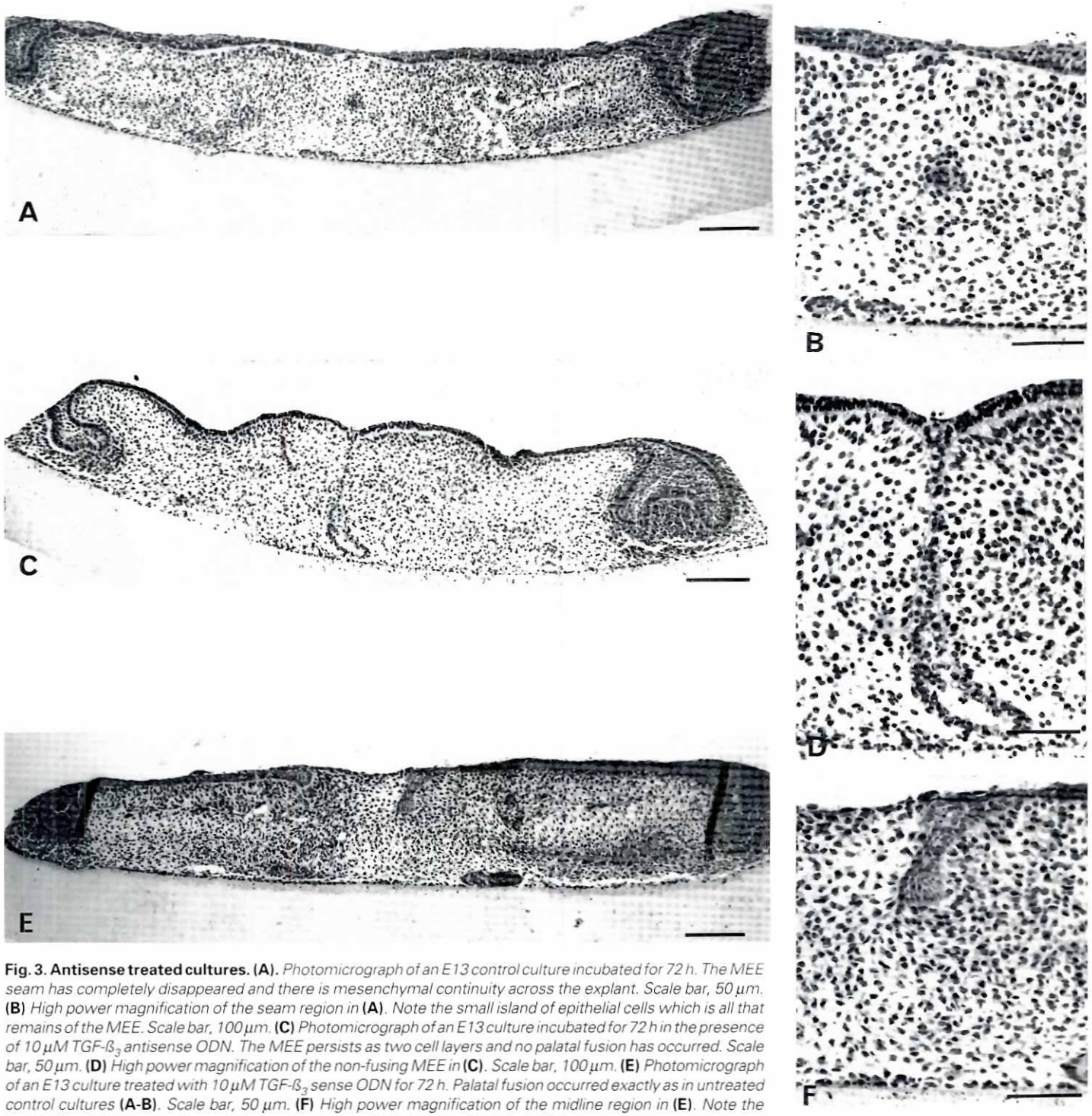

Fig. 3. Antisense treated cultures. (A). *Photomicrograph of an E13 control culture incubated for 72 h. The MEE seam has completely disappeared and there is mesenchymal continuity across the explant. Scale bar, 50 μm.* **(B)** *High power magnification of the seam region in* **(A)**. *Note the small island of epithelial cells which is all that remains of the MEE. Scale bar, 100 μm.* **(C)** *Photomicrograph of an E13 culture incubated for 72 h in the presence of 10 μM TGF-ß$_3$ antisense ODN. The MEE persists as two cell layers and no palatal fusion has occurred. Scale bar, 50 μm.* **(D)** *High power magnification of the non-fusing MEE in* **(C)**. *Scale bar, 100 μm.* **(E)** *Photomicrograph of an E13 culture treated with 10 μM TGF-ß$_3$ sense ODN for 72 h. Palatal fusion occurred exactly as in untreated control cultures* **(A-B)**. *Scale bar, 50 μm.* **(F)** *High power magnification of the midline region in* **(E)**. *Note the continuity of the mesenchyme between the two palatal shelves. Scale bar, 100 μm.*

doses of each antisense oligodeoxynucleotide (antisense ß$_1$, ß$_2$ or ß$_3$) higher than 20 μM resulted in regionalized epithelial cell death suggesting toxicity to the explant.

Treatment of palate cultures with a sense TGF-ß$_3$ ODN resulted in normal palate fusion (Figs. 2E-F, 3E-F). Similarly, treatment with various doses up to 10 μM of antisense TGF-ß$_1$ and TGF-ß$_2$ ODNs had no effect on palate development and the cultures fused in the same way as untreated controls (Table 1).

Northern analysis of antisense-treated cultures

Northern analysis was performed on RNA extracted from embryonic palatal shelves, control and ODN treated organ cultures for

TABLE 1

CUMULATIVE RESULTS OF THE EFFECT OF ANTISENSE ODN TREATMENT ON PALATAL SEAM FUSION *IN VITRO*

	Time of culture (h)	
	48	72
Treatment		
Control	5	99
Antisense -ß1	0	97
Antisense -$ß_2$	0	97
Antisense -ß3	0	6
Antisense -ß3 + TGF-$ß_3$ (10 ng/ml)	0	45
TGF-ß3 (10 ng/ml)	95	100

Each value indicates the percentage of total cultures which have undergone complete seam fusion and represents 3 independent experiments each of which used 10 cultures/time point/treatment.

TGF-$ß_3$ mRNA (Fig. 4). A peak of TGF-$ß_3$ expression has been reported *in vivo* at the time of MEE formation, i.e. early embryonic day 14 (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). In palatal organ culture normal development is delayed by approximately 24 h (Brunet *et al.*, 1993), so that TGF-$ß_3$ expression would be expected to occur after approximately 24-36 h of culture of embryonic day 13 explants. Northern analysis confirmed that this was the case (Fig. 4). There was a considerable reduction in the amount of TGF-$ß_3$ mRNA in antisense ODN TGF-$ß_3$-treated cultures compared to the same stage control and sense TGF-$ß_3$ ODN-treated cultures (Fig 4). Treatment with antisense ODN to TGF-$ß_1$ and TGF-$ß_2$ had no effect on TGF-$ß_3$ mRNA expression.

Time window of effectiveness of antisense ODN to TGF-$ß_3$

Further experiments were performed to establish the time period during which TGF-$ß_3$ must be active for normal palate fusion to occur. Palatal shelves were cultured for 72 h and treated with antisense ODN to TGF-$ß_3$ for various times; 0-72 h, 0-24 h, 36-72 h and 24-36 h. The results are presented in Fig. 5. This experiment confirmed that treatment with the antisense TGF-$ß_3$ ODN for the full period of culture blocked normal palatal fusion. However, if the ODN was added to the E13 palate cultures after 36 h, fusion occurred normally. Similarly, treatment with the ODN for 24 h and subsequent replacement with control medium resulted in normal palatal fusion. Therefore the antisense ODN to TGF-$ß_3$ appears to be required during the period of 24-36 h of culture in order to prevent fusion. Indeed, treatment of cultures with a single application of the antisense ODN to TGF-$ß_3$ at 24-36 h prevented palatal fusion. This period of sensitivity to the antisense ODN corresponds to the time window during which TGF-$ß_3$ gene expression is greatest. These experiments were supported by a similar organ culture experiment using embryonic day 12 palates. In such cultures MEE seam formation will normally occur between 48-72 h of culture, a 24 h delay when compared with embryonic day 13 cultures. Treatment of E12 cultures with antisense ODN to TGF-$ß_3$ over the entire period of culture prevented fusion (Fig. 5). However, the antisense ODN to TGF-$ß_3$ could be present for up to 48 h of culture before replacement with control medium and normal fusion still occurred.

Treatment of palate organ cultures with neutralizing antibodies

The use of antisense TGF-$ß_3$ suggested that TGF-$ß_3$ activity is vital for normal palate fusion. To confirm this, we intended to block TGF-$ß_3$ peptide activity using a neutralizing antibody. However, because of the high degree of similarity between TGF-ß isoforms, no neutralizing antibody specific to the native TGF-$ß_3$ isoform exists at the present time. Therefore, a panel of different TGF-ß isoform neutralizing antibodies were tested for their ability to prevent palatal fusion.

As expected from the antisense experiments, an antibody which neutralizes all the three TGF-ß isoforms blocked normal palate development (Figs. 6A-B, 7A-B). The appearance of cultures treated with 50 µg/ml of this antibody throughout incubation was very similar to the antisense TGF-$ß_3$ ODN-treated cultures. After 48 h of culture, the MEE had not adhered and no midline seam was formed (Fig. 6A-B). After 72 h, the cultures had not fused and a thick midline seam persisted (Fig. 7A-B).

In support of a specific role for TGF-$ß_3$, neutralizing antibodies specific to TGF-$ß_1$ or TGF-$ß_2$ had no effect on palate fusion and cultures appeared exactly as untreated controls (data not shown).

Surprisingly, however, an antibody which was described by the manufacturers (Genzyme, MA, USA) as one which neutralized TGF-$ß_2$ and TGF-$ß_3$ also had no effect on palate fusion. We expected such a reagent to prevent palate fusion due to its inhibition of TGF-$ß_3$. We therefore investigated whether the published specificities of each of the antibodies were correct (Fig. 8).

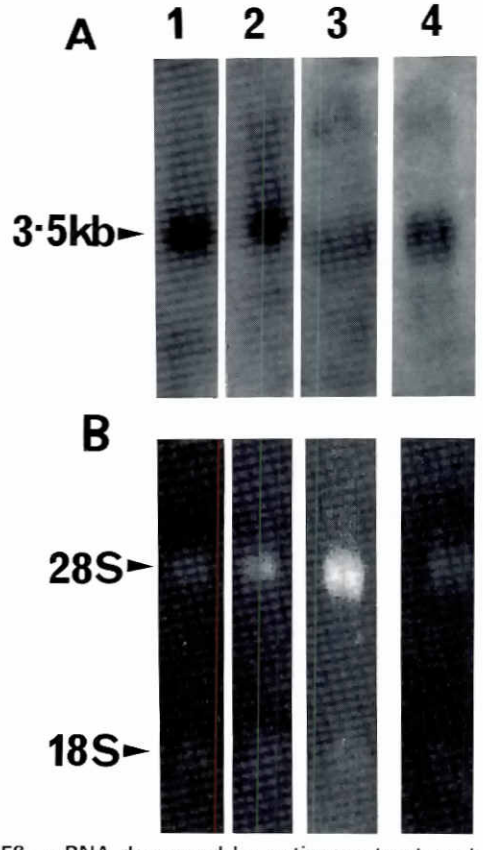

Fig. 4. TGFß$_3$ mRNA decreased by antisense treatment. (A) *Autoradiograph of a Northern blot hybridised with TGF-$ß_3$ probe. Lane 1 contains RNA from palates dissected on E14. Lane 2 contains RNA extracted from control palatal cultures at 24 h and lanes 3 and 4, RNA from antisense TGF-$ß_3$ ODN- and sense TGF-$ß_3$ ODN-treated cultures at 24 h respectively. Note the reduced level of TGF-$ß_3$ mRNA in the antisense treated cultures.* **(B)** *Ethidium bromide stained gel from which the accompanying autoradiograph was produced. All the samples shown come from one representative experiment and were electrophoresed on the same*

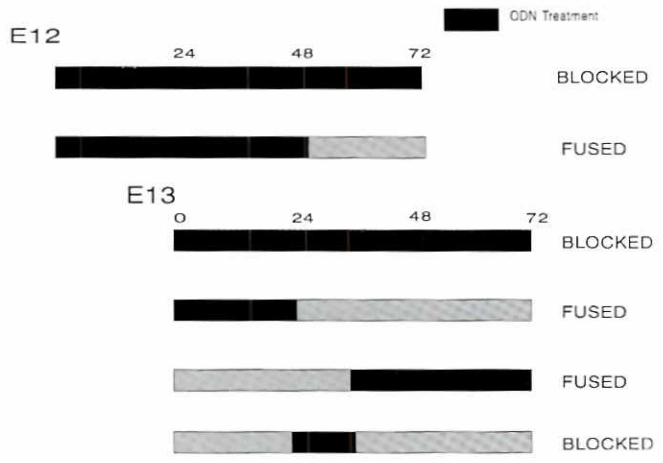

Fig. 5. The effect of various timed exposures to TGF-ß$_3$ antisense ODN on palatal fusion *in vitro*. *Palatal shelves were explanted on E12 and E13 and cultured. Each culture was incubated for 72 h in the presence or absence of TGF-ß$_3$ antisense for some or all of that time. Fusion was determined by routine histology and light microscopy. Each result represents analysis of 10 cultures.*

The pan-neutralizing antibody was effective in neutralizing 100% of the activity of half maximal concentrations of TGF-ß$_1$, ß$_2$, and ß$_3$ (Fig. 8A). The antibodies reported to be specific in neutralizing TGF-ß$_1$ and TGF-ß$_2$ activity proved to be so (Fig. 8B-C). However, the Genzyme antibody, previously reported to neutralize both TGF-ß$_2$ and ß$_3$, was effective in neutralizing only approximately 60-70% of the activity of each of these isoforms (Fig. 8D). As a consequence, this antibody appeared to have had insufficient neutralizing activity against TGF-ß$_3$ *in vitro* to prevent palate fusion. Moreover, contrary to the product information, we observed that this antibody also markedly neutralized TGF-ß$_1$ at low concentrations (Fig. 8D). At higher doses steric interference may result in the lack of an effect on TGF-ß$_1$ activity. Use of the antibody at only high concentrations in previous bioassays may account for the reported lack of TGF-ß$_1$ neutralizing activity of this antibody.

Rescue of normal fusion by adding TGF-ß$_3$

Addition of TGF-ß$_3$ (10 ng/ml) at the same time as the antisense ODN rescued palate fusion in some, but not all experimental cultures. This result was surprising, but in several repeats of these experiments the best recovery of palatal fusion in the presence of TGF-ß$_3$ ODN by added TGF-ß$_3$ was 60%. However, all cultures treated with TGF-ß$_3$ at the same time as the anti-ß$_1$, -ß$_2$, -ß$_3$ antibody fused normally (Figs. 6C-D, 7C-D).

Discussion

The process of mammalian palate fusion involves adhesion of the medial edge epithelia of apposing shelves to form an epithelial seam, followed by migration of the epithelial seam cells into oral and nasal triangles (Carette and Ferguson, 1992) and perhaps their transformation into mesenchyme (Fitchett and Hay, 1989; Shuler *et al.*, 1991, 1992; Griffith and Hay, 1992). This study investigated the roles of individual TGF-ß isoforms in this process. Inhibition of TGF-ß$_3$ activity prevented palatal fusion, whereas inhibition of the other TGF-ß isoforms had no effect on fusion. The prevention of fusion was the result of the ability of the antisense ODN and antibody to specifically inhibit expression and activity of TGF-ß$_3$. This was confirmed by Northern analysis which demonstrated that levels of TGF-ß$_3$ mRNA were reduced in antisense TGF-ß$_3$ treated cultures. Ideally, to confirm that the TGF-ß$_3$ ODN specifically targeted TGF-ß$_3$ production we would have liked to assess TGF-ß$_3$ protein levels in our cultures. However, conventional analysis by western blot, ELISA or immunohistochemistry is unavailable because of the lack of a suitable antibody which specifically recognizes native TGF-ß$_3$ and does not crossreact with other isoforms.

That other ODN constructs and antibodies had no effect on TGF-ß$_3$ expression and fusion provided further evidence that the effects seen were due to a specific inhibition of TGF-ß$_3$ activity. Moreover, the developmental time window for antisense ODN activity in preventing palatal fusion corresponded precisely with the peak of TGF-ß$_3$ mRNA expression. In neutralization experiments, addition of exogenous TGF-ß$_3$ allowed normal fusion to continue in the antibody-, and some but not all ODN-, treated tissues. The results from the antibody experiments can be simply explained by saturation of the antibody by exogenous TGF-ß$_3$ thus preventing effective neutralization of endogenous TGF-ß$_3$. By contrast, in cultures treated with antisense ODN, expression of endogenous TGF-ß$_3$ was inhibited but the exogenous TGF-ß3 was only able to rescue fusion in 60% of cultures. This result was surprising but there are several possible explanations. The exogenous TGF-ß$_3$ protein may have been at the wrong physiological concentration or not in the correct spatial and temporal distribution. Addition of excess TGF-ß$_3$ throughout the experiment may have down-regulated the specific type I/type II TGF-ß receptor profile rendering the cells non-responsive to their normal signal. Whether such down-regulation occurs is controversial and appears to depend on the cell type and culture conditions (Frolik *et al.*, 1984; Massague and Like, 1985; Wakefield *et al.*, 1987). Finally, it may be that recombinant TGF-ß$_3$ has different biological activity to that of the native form.

Timed exposure of E13 palatal cultures to antisense TGF-ß$_3$ revealed that the critical time for TGF-ß$_3$ expression was between 24 and 36 h, precisely when fusion occurs. These data suggest that TGF-ß$_3$ activity is vital for normal palate fusion.

How TGF-ß$_3$ regulates the complex process of palatal fusion is unknown. Fusion is a multi-step phenomenon, with presumably several signalling molecules involved. An early event is the coming together and adhesion of the two palatal shelves. Subsequently, the MEE of the two shelves merge to form a midline seam. Finally, the MEE cells disperse leading to mesenchymal continuity (Ferguson, 1988). One could envisage TGF-ß3 being involved in one or several of these steps.

TGF-ß$_1$ has been shown to stimulate the production of desmosomes in bronchial epithelial cells (Yoshida *et al.*, 1992). Desmosomal junctions have been implicated in the initial adhesion between the two palatal shelves (Morgan and Pratt, 1977). The increase in TGF-ß$_3$ expression in the MEE just prior to contact (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990) may serve to up-regulate desmosome formation at the apical and lateral surfaces of these cells. This would explain the lack of adhesion between palatal shelves in cultures treated with antisense ODN or neutralizing antibody against TGF-ß$_3$. Excess TGF-ß$_1$, -ß$_2$ or -ß$_3$ may stimulate desmosome formation earlier than in control cultures leading to accelerated adhesion (Dixon and Ferguson, 1992; Gehris and Greene, 1992; Brunet *et al.*, 1993). If this adhesion between the two shelves then goes on to signal subsequent fusion events (Brunet

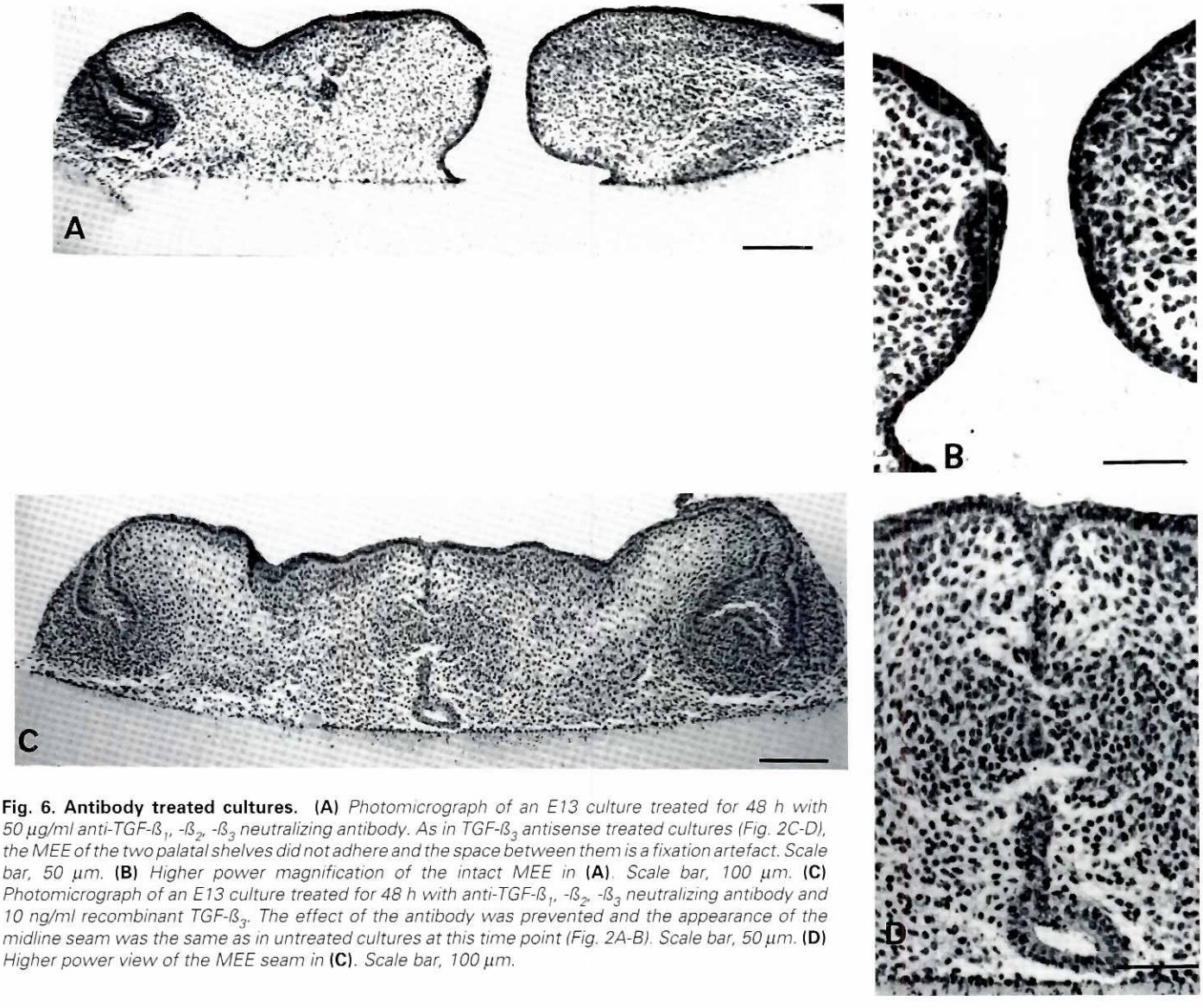

Fig. 6. Antibody treated cultures. **(A)** *Photomicrograph of an E13 culture treated for 48 h with 50 µg/ml anti-TGF-ß$_1$, -ß$_2$, -ß$_3$ neutralizing antibody. As in TGF-ß$_3$ antisense treated cultures (Fig. 2C-D), the MEE of the two palatal shelves did not adhere and the space between them is a fixation artefact. Scale bar, 50 µm.* **(B)** *Higher power magnification of the intact MEE in* **(A)**. *Scale bar, 100 µm.* **(C)** *Photomicrograph of an E13 culture treated for 48 h with anti-TGF-ß$_1$, -ß$_2$, -ß$_3$ neutralizing antibody and 10 ng/ml recombinant TGF-ß$_3$. The effect of the antibody was prevented and the appearance of the midline seam was the same as in untreated cultures at this time point (Fig. 2A-B). Scale bar, 50 µm.* **(D)** *Higher power view of the MEE seam in* **(C)**. *Scale bar, 100 µm.*

et al., 1993), this would explain why fusion is accomplished earlier in TGF-ß treated cultures. Certainly in the ODN and antibody treated cultures the critical problem seems to be a lack of adherence between the MEE of the two shelves.

At a later stage of palatal fusion, TGF-ß$_3$ may stimulate the trans-differentiation of the MEE cells. Interestingly, TGF-ß has been shown to induce mammary epithelial cells to transform to mesenchyme (Miettinen *et al.*, 1994). Similarly, a role for TGF-ß$_3$ has previously been demonstrated in the epithelial-mesenchymal transformation which occurs during normal heart valve development (Potts *et al.*, 1991; Runyan *et al.*, 1992). Perhaps this particular TGF-ß isoform plays a similar role in other such embryonic epithelial-mesenchymal transformations for example in the developing lung and kidney.

TGF-ß$_3$ may stimulate the migration of MEE cells away from the midline (Carette and Ferguson 1992). This may be via its effects on the cells themselves (e.g. acquisition of a migratory «mesenchymal» phenotype), or on their cell adhesion molecules, or on the composition of the underlying extracellular matrix. TGF-ß$_1$ is known to stimulate the accumulation of specific extracellular matrix molecules (Seyedin *et al.*, 1985, 1986; Ignotz *et al.*, 1987; Rossi *et al.*, 1988; Romaris *et al.*, 1991), and significantly ECM production by palate mesenchymal cells (Sharpe *et al.*, 1992b) and whole palate organ cultures (Foreman *et al.*, 1991).

Finally, inhibition of TGF-ß$_3$ activity may have more indirect effects. Because of the interplay between several different growth factors and their receptors, removal of just one of the elements in the signalling cascade may disrupt the whole system. For example, TGF-ß$_1$ has been shown to down-regulate EGF receptors in palate mesenchyme cells (Sharpe *et al.*, 1992a) and palatal epithelia (Brunet *et al.*, 1993). Such down-regulation may be necessary to prevent EGF or TGF-α activity. Both EGF and TGF-α prevent normal palatal fusion (Abbott and Pratt, 1987). Removal of TGF-ß$_3$ activity by antisense or antibody may prevent palatal fusion by allowing EGF or TGF-α to be active in the medial edge region at inappropriate times. TGF-α has been suggested to be a 'candidate'

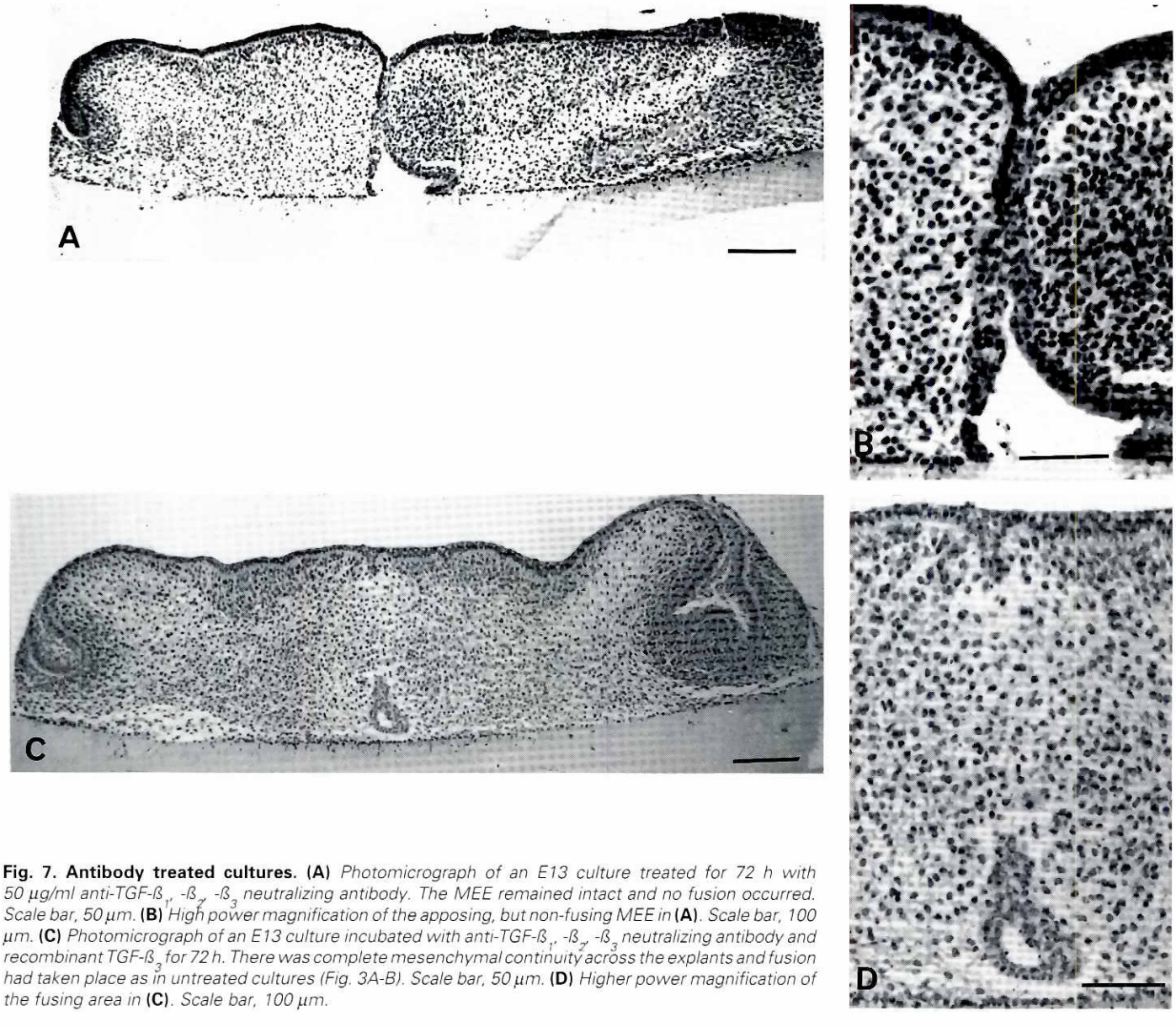

Fig. 7. Antibody treated cultures. (A) *Photomicrograph of an E13 culture treated for 72 h with 50 μg/ml anti-TGF-ß$_1$, -ß$_2$, -ß$_3$ neutralizing antibody. The MEE remained intact and no fusion occurred. Scale bar, 50 μm.* **(B)** *High power magnification of the apposing, but non-fusing MEE in* **(A)**. *Scale bar, 100 μm.* **(C)** *Photomicrograph of an E13 culture incubated with anti-TGF-ß$_1$, -ß$_2$, -ß$_3$ neutralizing antibody and recombinant TGF-ß$_3$ for 72 h. There was complete mesenchymal continuity across the explants and fusion had taken place as in untreated cultures (Fig. 3A-B). Scale bar, 50 μm.* **(D)** *Higher power magnification of the fusing area in* **(C)**. *Scale bar, 100 μm.*

gene which is disrupted in human cleft palate: an association between a specific TGF-α RFLP and susceptibility to human facial clefting has been demonstrated by several groups (Ardinger *et al.*, 1989; Chevenix-Trench *et al.*, 1991). Our present data highlight TGF-ß$_3$ as another candidate gene for disruption in human facial clefting. This finding is especially interesting since it has been shown that in a transgenic mouse in which TGF-ß$_1$ expression is prevented, facial development is normal, but a transgenic in which TGF-ß$_3$ expression has been blocked has cleft palate as its only phenotype (G. Proetzel and T. Doetschman, personal communication).

A significant finding of this study is that there is a functional diversity in the role of TGF-ß isoforms in regulating embryonic development.

Much of the early work on the biological activity of TGF-ß used only TGF-ß$_1$ since the other isoforms had not been discovered. It was assumed that since all three isoforms bound to the same receptors they would have similar activity, but this may not be true. To date, several other studies comparing the three isoforms have revealed either quantitative or qualitative differences in activity (Ohta *et al.*, 1987; Jennings *et al.*, 1988; Rosa *et al.*, 1988; Graycar *et al.*, 1989; Cheifetz *et al.*, 1990; Joyce *et al.*, 1990). This isoform specificity may be determined by receptor subsets which bind with particularly high affinity to one TGF-ß isoform (Cheifetz *et al.*, 1990). Presumably the evolution of several TGF-ß isoforms allows greater flexibility and regulation of function. This is supported by the fact that each TGF-ß isoform, despite its highly conserved mature peptide, has a unique upstream regulatory sequence and promoter (Roberts *et al.*, 1991). This results in the observed independent expression patterns (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). In addition, each isoform has a unique precursor region, the latency associated peptide (Derynck *et al.*, 1988; Ten Dijke *et al.*, 1988;

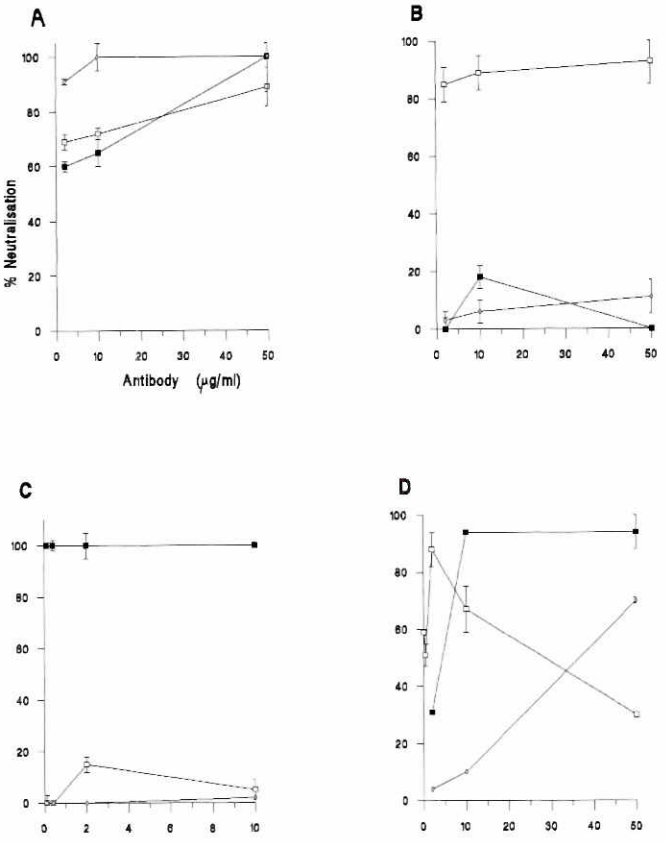

Fig. 8. Antibody specificities for TGFß isoforms. *Neutralization of TGF-$ß_1$ (1 ng/ml, ❑), TGF-$ß_2$ (1 ng/ml, ■) and TGF-$ß_3$ (0.5 ng/ml, ○) by anti-$ß_1$,$ß_2$,$ß_3$* **(A)**, *anti-$ß_1$* **(B)**, *anti-$ß_2$* **(C)** *and anti-$ß_2$,$ß_3$* **(D)** *antibodies. The antibodies were preincubated with the TGF-ß isoforms at the indicated concentrations, as described in Materials and Methods, before being added to Mv1Lu cells. The effect on proliferation was assessed using the ^{3}H-thymidine incorporation assay and the results expressed as percentage neutralization of the effect of TGF-$ß_1$, -$ß_2$ or -$ß_3$. Each point represents the mean (±SEM) of three separate determinations.*

Roberts and Sporn, 1990), which may ensure isoform-specific activation. The fact that each TGF-ß isoform has been highly conserved throughout evolution compared to the degree of conservation between isoforms suggests that the system does not necessarily have a high level of redundancy *in vivo*. More likely, the duplication of an ancestral TGF-ß gene allowed for the evolution of isoform specific effects.

Our data provide evidence for an important functional role of TGF-$ß_3$ in palatogenesis, independent of the actions of TGF-$ß_1$ and $ß_2$. Our data also support earlier findings in the heart (Potts *et al.*, 1991; Runyan *et al.*, 1992) and suggest that the TGF-$ß_3$ isoform plays a unique role in embryonic development, possibly by regulating the processes of epithelial adhesion, transformation and migration.

Materials and Methods

In vitro palate organ culture

Palate organ cultures were established as described in Brunet *et al.* (1993). Briefly, palatal shelves were dissected from embryonic day 13 mouse embryos (Manchester strain, MF1; Theiler (1972) stage 21) and placed in pairs on 0.8 µm Millipore filters with their MEE in close apposition to simulate the *in vivo* orientation. The cultures were allowed to attach to the filters in a conventional Trowell organ culture system containing Minimal Essential Medium (Flow Laboratories, Irvine, Scotland, UK) supplemented with 1% glutamine, at 37°C in an air incubator. After 6 h the cultures were submerged in 20 µl DMEM/F12 (Dulbecco's Minimal Essential Medium/Ham's F12 Growth Medium; Flow Laboratories, Irvine, Scotland, UK) supplemented with 1% glutamine, 40 µg/ml ascorbate and 1% penicillin/streptomycin at 37°C in a 5% CO_2 environment. Cultures were then treated with either antisense ODN (10 µM) or neutralizing antibodies (50 µg/ml) to TGF-ß isoforms with 10 cultures per time point per treatment. In addition, 10 cultures were treated with either the antisense TGF-$ß_3$ ODN or neutralizing antibody in the presence of exogenous recombinant TGF-$ß_3$ (10 ng/ml, British Biotechnology, Oxford, UK). The medium was changed every 12 h and the cultures were fixed in 4% paraformaldehyde at 24, 48 and 72 h before being processed for wax histology. Sections were stained using alcian blue, Harris' hematoxylin and eosin and photographed. Each experiment was repeated a minimum of three times.

Source of antisense ODN and neutralizing antibodies

Unmodified, phosphodiester, 19 base oligodeoxynucleotides were obtained from Zeneca Pharmaceuticals, UK and Oligo's Etc. Inc., USA. The sequences used flank the translation start site of the published cDNAs encoding the TGF-ß isoforms (Derynck *et al.*, 1986; Miller *et al.*, 1989a,b).

TGF-$ß_1$ Antisense	5' gAg ggC ggC ATg ggg gAg g 3'
TGF-$ß_2$ Antisense	5' CAC ACA gTA gTg CAT gTT T 3'
TGF-$ß_3$ Antisense	5' CCT TTg CAA gTg CAT CTT C 3'
TGF-$ß_3$ Sense	5' gAA gAT gCA CTT gCA AAg g 3'

The neutralizing antibodies used were as follows: Anti TGF-$ß_1$ (Chicken IgG), British Biotechnology, Oxford, UK; Anti TGF-$ß_2$ (Rabbit IgG), British Biotechnology, Oxford, UK; Anti TGF-$ß_1$, -$ß_2$, -$ß_3$ (Monoclonal mouse IgG), kind gift from Genzyme, Cambridge, MA, USA; Anti TGF-$ß_2$, -$ß_3$ (Monoclonal mouse IgG), kind gift from Genzyme, Cambridge, MA, USA.

Northern blot analysis

RNA was extracted from freshly dissected palates (10 pairs) from E14 embryos or control/antisense ODN treated organ cultures (10 cultures/time point) in 4 M lithium chloride using an Ultrasonics, Inc. sonicator. Samples of RNA (10 µg) were electrophoresed through a 1.2% denaturing agarose gel. The amount of RNA loaded was quantified by ethidium bromide staining (Fig. 4B). RNA was then blotted overnight onto a nylon membrane (Amersham, Hybond-N). Hybridizations were carried out at 42°C in 50% formamide, 5XSSC, 0.2% SDS, 5XDenhardt's reagent, 300 µg/ml tRNA, for 15-20 h using ^{32}P-labeled TGF-$ß_3$ cDNA (kind gift from Dr. A. Roberts, NIH, Bethesda, USA). After washing, filters were exposed to Kodak XAR-2 film at -70°C for 3-7 days.

Antibody specificity assay

The specificity of the neutralizing antibodies was assessed using a variation of the Mv1Lu cell ^{3}H-Thymidine incorporation assay described in Sharpe *et al.* (1992a). Briefly, the antibodies at varying concentrations (0.1-50 µg/ml) were incubated for 1 h at 37°C in the presence of TGF-$ß_1$ (1 ng/ml), -$ß_2$ (1 ng/ml) or -$ß_3$ (0.5 ng/ml) (British Biotechnology, Oxford, UK). The TGF-ß isoform concentrations were pre-determined to represent half maximal effectiveness in down regulating Mv1Lu proliferation. The antibody/TGF-ß solutions were then added to the Mv1Lu cells in DMEM/F12 supplemented with 1% glutamate, 40 µg/ml ascorbate and 2% donor calf serum (Gibco BRL, Uxbridge, UK), and incubated for 24 h. For the last 2 h of incubation [6-^{3}H]-thymidine (Amersham, specific activity 29 Ci/mmol) was added to the medium (1 µCi/ml). Cells were fixed in 3 washes with 5% TCA and remaining radioactivity was solubilized with 0.5 ml of NaOH (0.5 M) at 37°C for 1 h. Counts were detected using a Beckman 9800 scintillation counter.

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