Development 126, 3981-3989 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV9657

Regeneration of isthmic tissue is the result of a specific and direct interaction between rhombomere 1 and midbrain

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Accepted 19 June; published on WWW 23 August 1999

SUMMARY

The midbrain-hindbrain boundary, or isthmus, is the source of signals that are responsible for regional specification of both the midbrain and anterior hindbrain. Fibroblast growth factor 8 (Fgf8) is expressed specifically at the isthmus and there is now good evidence that it forms at least part of the patterning signal. In this study, we use *Fgf8* as a marker for isthmic cells to examine how interactions between midbrain and hindbrain can regenerate isthmic tissue and, thereby, gain insight into the normal formation and/or maintenance of the isthmus. We show that *Fgf8*-expressing tissue with properties of the isthmic organiser is generated when midbrain and rhombomere 1 tissue are juxtaposed but not when midbrain contacts any other rhombomere. The use of chick/quail chimeras shows that the isthmic tissue is largely derived from rhombomere 1. In a few cases a small proportion of the *Fgf8*-positive cells were of midbrain origin but this appears to be the result of a local respecification to a hindbrain phenotype, a process mimicked by ectopic FGF8. Studies in vitro show that the induction of *Fgf8* is the result of a direct planar interaction between the two tissues and involves a diffusible signal.

Key words: Isthmus, Boundary, Hindbrain, Midbrain, Rhombomere, Fgf8, Gbx2, Cell signalling, Regeneration

INTRODUCTION

The isthmus is the morphological boundary between midbrain and hindbrain. It is also an organising centre within the developing neuroepithelium that provides a source of planar signals to pattern both the midbrain rostrally and the anterior hindbrain caudally, along the anteroposterior (A-P) axis. Tissue grafting studies have shown that isthmic signals can respecify anterior midbrain to a posterior midbrain phenotype and can respecify posterior hindbrain to a cerebellar fate (characteristic of the hindbrain region adjacent to the isthmus). More dramatically, it can cause posterior forebrain tissue to develop as an ectopic midbrain (reviewed in Joyner 1996; Bally-Cuif and Wassef, 1995; Wassef and Joyner, 1997). The differential competence of hindbrain and midbrain to respond to the isthmic signal(s) is reflected by expression domains of *Otx2* (forebrain and midbrain) and *Gbx2* (hindbrain) which abut at the isthmus and are expressed from the time of neural induction (Bally-Cuif et al., 1995; Shamim and Mason, 1998).

The isthmic organising signal is thought to be provided by signalling molecules that establish a gradient of positional information, reflected in the graded expression of genes such as *En2*. The secreted signalling protein, fibroblast growth factor 8 (Fgf8), is expressed specifically at the isthmus at developmental stages 10-12; (Hamburger and Hamilton, 1951; HH stage 10-12) used in the grafting studies which first established isthmic function (Crossley et al., 1996; Shamim et al., 1999). Beads soaked in FGF8 protein can mimic isthmic tissue grafts when implanted into the avian midbrain, as evinced by the ectopic induction of posterior midbrain markers and production of ectopic, polarised midbrain structures (Crossley et al., 1996; Sheikh and Mason, 1996; Shamim et al., 1999; Martinez et al., 1999). In addition, FGF8 beads placed into posterior rhombomeres (r) induce molecular characteristics specific to rhombomere 1 (r1; C. I. and I. M. unpublished observations). *Fgf8* expression at the midbrain-hindbrain boundary is conserved in all vertebrate classes (Crossley and Martin, 1995; Mahmood et al., 1995a; Crossley et al., 1996; Christen and Slack, 1997; Riefers et al., 1998). In mice, ectopic expression of Fgf8 in the midbrain produces similar, but not identical, results to the experiments in avian embryos using beads (Lee et al., 1997 and see also Shamim et al., 1999 for discussion). Moreover, while null mutations of *Fgf8* in mice are embryonic lethal due to gastrulation defects, hypomorphic alleles reveal that *Fgf8* is an essential component of the isthmus organiser and required for the normal development of both cerebellum and posterior midbrain structures (Meyers et al., 1998). Furthermore, zebrafish acerebellar (ace) mutants in which Fgf8 is either partially or completely inactivated also lack a cerebellum, isthmus and posterior midbrain structures (Reifers et al., 1998).

Specialised boundary cells arise not only at the isthmus but also between rhombomeres within the hindbrain, although only

the former express *Fgf8*. However, previous studies have provided considerable insight into inter-rhombomeric boundary generation and maintenance. Prior to boundary formation, cells in the hindbrain become progressively restricted within rhombomeric domains (Irving et al., 1996a). A two segment periodicity within the rhombomeres ensures that neighbouring groups of cells separate due to adhesive differences and repulsive interactions (Guthrie and Lumsden, 1991; Xu et al., 1995). Any cells that cross into adjacent territories may become reprogrammed to a new molecular identity characteristic of their new rhombomeric location along the A-P axis (reviewed by Irving et al., 1996b). Subsequently, a distinct population of boundary cells arises at the interface between adjacent rhombomeres. These cells aid the isolation of adjacent rhombomeres and are characterised by a low rate of proliferation, a distinct portfolio of molecular markers and a specialised cellular architecture (Lumsden and Keynes, 1989; Guthrie et al., 1991; Heyman et al., 1993, 1995; Mahmood et al., 1995b). Loss of gap-junctional communication between neighbouring rhombomeres also occurs across boundaries and this may further contribute to the establishment and/or maintenance of individual rhombomere identities (Martinez et al., 1992).

Rhombomere boundaries regenerate following ablation, and transplantation studies reveal that boundaries form when odd-numbered rhombomeres are grafted next to even-numbered rhombomeres but not when odd-odd or even-even combinations are generated (Guthrie and Lumsden, 1991; Mahmood et al., 1995b). This process may be mediated by signalling between members of the eph and ephrin families (Xu et al., 1995). By contrast, little is known about the formation and maintenance of the isthmus, which differs from boundaries between rhombomeres in its organiser properties, *Fgf8* expression and because gap-junctional communication is not restricted across this boundary (see above and Martinez et al., 1992). We have investigated the regeneration of isthmic tissue following ablation and in heterotopic grafts of midbrain and hindbrain tissue. We confronted midbrain tissue with rhombomeres from different axial levels and compared their ability to generate tissue with isthmic characteristics. Heterospecific, heterotopic grafts were used to investigate the origin of regenerated isthmic cells and in vitro approaches employed to study the nature of the interaction and the signal.

MATERIALS AND METHODS

Removal of the isthmus by aspiration

A domain encompassing the isthmic constriction was removed from HH stage 10 chick embryos as described for rhombomere boundaries by Guthrie and Lumsden (1991) and Mahmood et al. (1995b). The boundary region was aspirated in ovo using a microelectrode attached to a mouth pipette. Eggs were sealed with tape and either incubated for a further 24 hours or selected randomly as controls.

Tissue transplantations

Donor chick or quail embryos were incubated to HH stage 10-11, dissected in Howard's Ringer and pinned out on a Sylgard- (Dow-Corning) coated dish. To mark polarity, small focal injections of DiI C_{12} (Molecular probes; 5 mg/ml in dimethyl formamide) were made into the anterior of the region to be grafted. The neural tube was excised and treated with Dispase I (Boehringer Mannheim) 1 mg/ml in L-15 medium (Life Technologies) containing 5 μg/ml DNAse I (Boehringer Mannheim) for 5 minutes to separate the neural tube and surrounding mesenchymal cells. The latter were then mechanically dissected away using a tungsten needle. The graft region was removed by further microdissection of either the left or right side of the neural tube and transplanted into stage-matched hosts in ovo.

Host chick embryos were 'windowed' and visualised by a sub-blastodermal injection of India ink. The vitelline membrane over the graft site was removed and tissue from the appropriate location for insertion of the graft was removed by microdissection using tungsten needles. The graft tissue was introduced with a serum-coated micropipette and manoeuvred into place. Eggs were sealed with tape and incubated for a further 24 hours prior to in situ hybridisation or for 7-8 days prior to fixation, sectioning and staining with cresyl violet as described by Shamim et al. (1999).

Co-cultures of explants in collagen gels

Neural tubes from HH stage 10-11 chick embryos were isolated from associated tissue as described for grafts. Four incisions were made across the neural tube using tungsten needles; these were at the levels of anterior midbrain, posterior midbrain, anterior r1 and posterior r1. Thus, 3 pieces of tissue were collected: midbrain tissue, r1 tissue, and a control region containing the isthmus. Explants were embedded either alone or juxtaposed in a bovine dermal collagen matrix (Cellon) which was set by adjusting its pH (Shamim et al., 1999). Explants were cultured for 24 hours in OPTIMEM medium (Life Technologies) supplemented with N2 serum-free supplement (Life Technologies) in a humidified atmosphere of 5% (v/v) carbon dioxide at 37°C. For trans-filter cultures, midbrain and r1 tissue were held in place in collagen gels either side of a 3 μm or 0.4 μm pore size Nuclepore filter (Millipore).

Double whole-mount in situ hybridisation and immunohistochemistry

Whole-mount in situ hybridisation of embryos was performed as described by Shamim et al. (1999) using probes which have been previously reported (Shamim and Mason, 1998; Shamim et al., 1999). In situ hybridisation of collagen gel explants was performed as described by Henrique et al. (1995).

Following in situ hybridisation embryos were post-fixed in 4% paraformaldehyde for 20 minutes, then whole-mount immunohistochemistry using the quail-specific antibody, QCPN (Hybridoma bank, Iowa University, Iowa, USA), and either a peroxidase- or a fluorescently labelled secondary antibody was performed to visualise the grafted cells (Mason, 1999).

Implantation of FGF beads

Implantation of FGF8-soaked beads was performed as described by Shamim et al. (1999).

RESULTS

Neuromere boundaries form in a specific sequence between HH stage 9– and HH stage 12 and the morphological isthmic constriction is evident from HH stage 9– (Vaage, 1969). All experiments in this study were performed between HH stage 10 and 11, when the isthmus and rhombomeres are clearly identifiable and isthmic *Fgf8* expression is established (Shamim et al., 1999).

Regeneration of isthmic tissue following ablation

We first investigated whether or not the isthmus was regenerated in a manner analogous to the rhombomeric boundaries in the hindbrain where recognition of cell surface

differences between two adjacent rhombomeric territories leads to boundary formation (see above). We performed ablation experiments to remove the isthmus and assess its ability to regenerate using in situ hybridisation for the presence of *Fgf8* transcripts as a marker of isthmic cells. An area encompassing and extending beyond the *Fgf8*-positive domain was aspirated with a micro-electrode (Fig. 1A). Embryos were allowed to develop for a further 24 hours, reaching between HH stage 14 and 17, by which time neural tissue had filled the ablation site. *Fgf8* expression was detected in a tight band within the regeneration site (n=20/22; Fig. 1B). Control embryos were selected randomly from among the operated embryos and assayed for *Fgf8* expression immediately after ablation, in order to ensure that all *Fgf8* positive cells had been removed. In all control cases, no *Fgf8* expression was observed in the midbrain-hindbrain region (n=0/14; Fig. 1C), but was still present in all other sites of expression.

Induction of *Fgf8* following grafts of r1 into midbrain

Regeneration of the isthmus following ablation may have been due to an inductive interaction arising from cellular differences between the adjacent midbrain and hindbrain tissue in the same way that the inter-rhombomeric boundaries are thought to be reformed (Guthrie and Lumsden, 1991). In order to address this question we grafted pieces of posterior r1 tissue unilaterally into the anterior midbrain (Fig. 2A). *Fgf8* is expressed in a tight band spanning the posterior midbrain and anterior r1 across the isthmic constriction at the stages used for grafting. Hence, only posterior r1 (i.e. *Fgf8*-negative) tissue was selected. To ensure that no *Fgf8*-positive 'isthmic' cells were transferred accidentally with the r1 graft, donor embryos were selected at random and assayed for *Fgf8* expression following removal of the r1 graft. In all cases, a region of *Fgf8*-negative cells was clearly visible between the isthmic expression domain and the excision point indicating that no isthmic cells had been transferred (Fig. 2B and data not shown).

Operated embryos were analysed after 24 hours for expression of *Fgf8*. When r1 was grafted into the midbrain, ectopic *Fgf8* mRNA was observed at the graft site (n=12/14; Fig. 2C and Table 1). Ectopic expression was restricted to the donor/host interface and was never seen to completely encompass the graft. Moreover, expression was generally observed only along one face of the graft.

Posterior rhombomere transplantations into midbrain fail to induce *Fgf8*

The isthmus forms at the boundary of *Gbx2* and *Otx2* expression domains; transcription factors that divide the neural plate into anterior and posterior territories from the time of its formation at HH stage 5, and may form a basis upon which patterning of this region is refined and the isthmus positioned (Bally-Cuif et al., 1995; Shamim and Mason, 1998). The induction of an ectopic isthmus (as marked by *Fgf8* expression) may be due to (i) a general interaction between midbrain (*Otx2*-positive) and anterior hindbrain (r1-3) cells (*Gbx2*-positive), (ii) an odd/even relationship analogous to that operating within the hindbrain or (iii) a specific interaction between midbrain and r1 cells. To address these possibilities posterior rhombomeres were grafted into the anterior midbrain.

Grafts of other rhombomeres were performed as described above for donor r1 into host midbrain. No difference was observed between grafts of r2 (n=16; Fig. 3A), r3 (n=11; Fig. 3B), r4 (n=5; Fig. 3C), r5 (n=6; Fig. 3D) or r6 (n=1; data not shown) into the midbrain: in all cases no ectopic induction of *Fgf8* was observed within the graft or in the surrounding host midbrain tissue (Fig. 3; Table 1).

Table 1. Summary of results of tissue grafting experiments

Type of graft	Gene expression	Total number of embryos	Integrated grafts	Embryos with ectopic gene expression
r1 to midbrain	*Fgf8*	14	14	12
r2 to midbrain	*Fgf8*	19	16	0
r3 to midbrain	*Fgf8*	14	11	0
r4 to midbrain	*Fgf8*	5	5	0
r5 to midbrain	*Fgf8*	9	6	0
quail r1 to midbrain	*Fgf8*	31	23	14
quail midbrain to r1	*Fgf8*	12	12	10
quail r1 to midbrain	*Wnt1*	10	10	8
quail r1 to midbrain	*Gbx2*	11	11	5

Fgf8 induction following transplantation of midbrain into hindbrain

In order to confirm that the induction of *Fgf8* was due to an interaction between the midbrain and r1 and to further ensure that there was no contamination by 'isthmic' cells in the r1 graft, an alternative strategy was employed. Tissue was excised

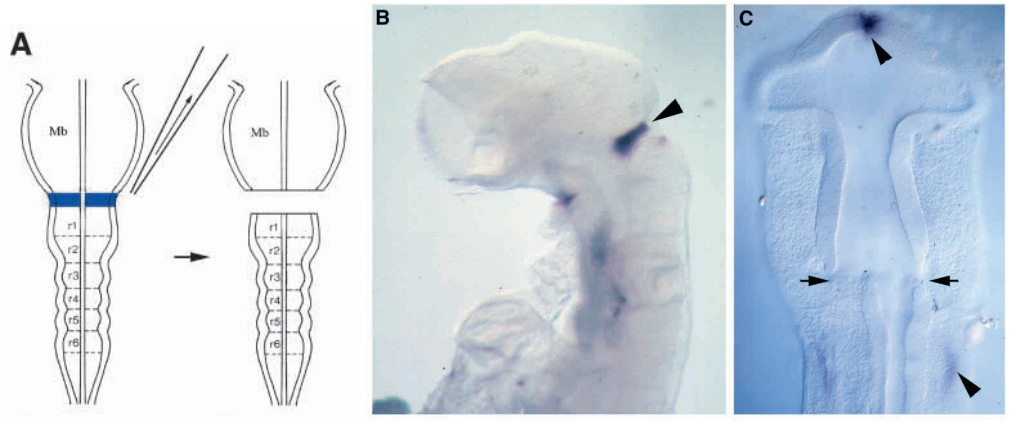

Fig. 1. *Fgf8* expression in regenerated isthmic tissue following ablation. Tissue, surrounding and including the isthmic constriction, was removed by aspiration as shown (A). Isthmic tissue is depicted in blue in this and all subsequent diagrams. (B)Embryos were analysed 24 hours later by in situ hybridisation for expression of *Fgf8* as a marker of isthmic cells. The arrowhead indicates a tight band of *Fgf8* in the regenerated tissue. (C) Control embryo fixed immediately after aspiration and probed for *Fgf8* expression demonstrates complete removal of *Fgf-8*-positive isthmic tissue (arrows) while expression is detected at the anterior neuropore and in branchial arches (arrowheads).

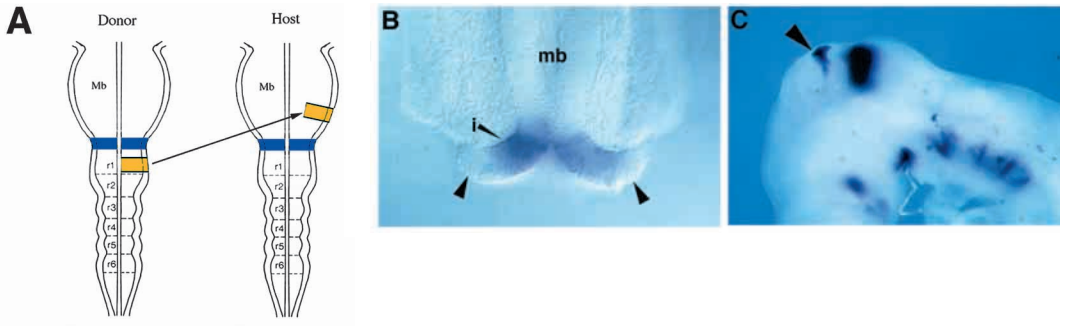

Fig. 2. *Fgf8* is induced following r1 grafts into midbrain. (A) Diagrammatic representation of the graft: posterior r1 (yellow) was transplanted heterotopically into host midbrain at HH stage 10. (B) In situ hybridisation to *Fgf8* on donor embryos after removal of posterior r1 ensured that no *Fgf8*-positive cells were transferred with the graft. The arrowheads indicate *Fgf8*-negative cells between the isthmic *Fgf8*-positive region and the excision. (C) In grafted embryos ectopic expression of *Fgf8* was observed at one face of the graft as indicated by the arrowhead; in this example ectopic expression is seen at the posterior side of the graft. i, isthmus; mb, midbrain.

from within the anterior half of donor midbrains and grafted unilaterally adjacent to posterior r1 of a host embryo. Due to the large size of the midbrain graft, the anterior edge of the graft was within posterior r1 and the posterior edge of the graft was within r2 (Fig. 4A).

Operated embryos were analysed for induction of *Fgf8* 24 hours later. In addition to the normal domain of *Fgf8* expression at the isthmus, a second stripe was seen between the midbrain graft and r1 (n=10/12; Fig. 4B,C and see Table 1). Ectopic *Fgf8* expression was always at the anterior boundary of the graft (i.e. at the interface with r1). By contrast, no *Fgf8* transcripts were detected at the posterior end of the graft where midbrain and r2 tissue were in contact (Fig. 4B,C), consistent with the results obtained when r2 was grafted into midbrain.

Fgf8 expression is induced by a direct interaction between midbrain and r1

From the tissue grafting experiments, it was unclear whether the interaction between the midbrain and r1 was due to direct planar signalling or also required surrounding tissues. To address this, we performed collagen explant co-cultures of isolated posterior r1 and anterior midbrain. Bilateral explants were excised from midbrain and posterior r1, treated with dispase and any remaining mesoderm and ectoderm removed by dissection. Explants were co-cultured in a collagen matrix for 24 hours (Fig. 5A), a time period equivalent to the transplantation studies, and assayed for *Fgf8* induction. When r1 and midbrain were juxtaposed within the collagen gel, *Fgf8* expression was induced at the interface of the two pieces of tissue and apparently spanned both explants (n=12/17; Fig. 5B); suggesting that the induction was a consequence of direct planar signalling between r1 and the midbrain. When r2 or r3 were juxtaposed with midbrain tissue in a collagen matrix *Fgf8* expression was never seen within the explants, confirming the earlier grafting studies in ovo (data not shown).

Again, it was essential to ensure that no *Fgf8*-positive cells were present in the explants when they were excised from the neural tube. We therefore also separately cultured the region from between the r1 and midbrain tissue pieces to show that this region contained all of the *Fgf8*-positive 'isthmic' cells. In all cases, the control isthmic region maintained a sharp band of *Fgf8* expression within the central domain of the explant which was flanked by *Fgf8*-negative cells on both sides (n=11/11; Fig. 5C). When cultured alone, r1 or midbrain explants never expressed *Fgf8*, but both retained molecular characteristics of their original position within the neural tube. r1 cultured alone retained its hindbrain phenotype as assayed by its continued expression of *Gbx2* and midbrain continued to express *Otx2* (data not shown).

A diffusible signal mediates the interaction between r1 and midbrain

To obtain evidence as to whether the inductive interaction between r1 and the midbrain was due to a diffusible molecule or was a cell-contact dependent process, co-cultured explants were separated by Nuclepore filters (Fig. 5D). r1 and midbrain explants were separated by either 3 μm filters, allowing both cell contact-dependent communication and the passage of diffusible molecules, or 0.4 μm filters, blocking direct cell contact-dependent signalling but allowing the passage of diffusible molecules. In both cases, *Fgf8* transcripts were detected in the tissue recombinants (n=11/12 for 3 μm filter; n=9/9 for 0.4 μm filter; data not shown and Fig. 5E,F). By contrast, no *Fgf8* transcripts were detected in similar transfilter

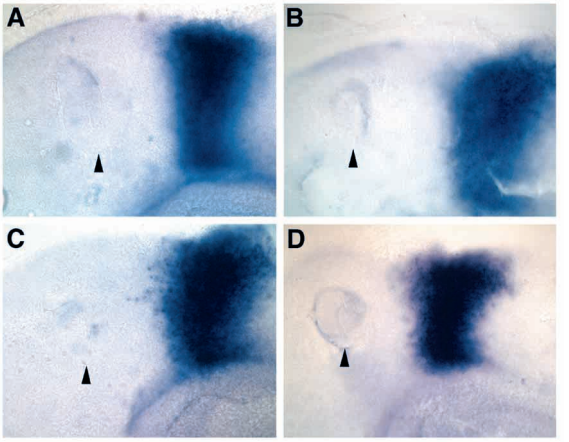

Fig. 3. *Fgf8* is not induced when posterior rhombomeres are grafted into midbrain. Grafts of posterior rhombomeres were made into midbrain and embryos were analysed for ectopic *Fgf8* expression by in situ hybridisation. Grafts of r2 (A), r3 (B), r4 (C) or r5 (D) failed to induce *Fgf8* expression either within the graft or the host midbrain, although normal *Fgf8* expression is observed at the isthmus. Locations of the grafts are indicated by arrowheads placed at their ventral side.

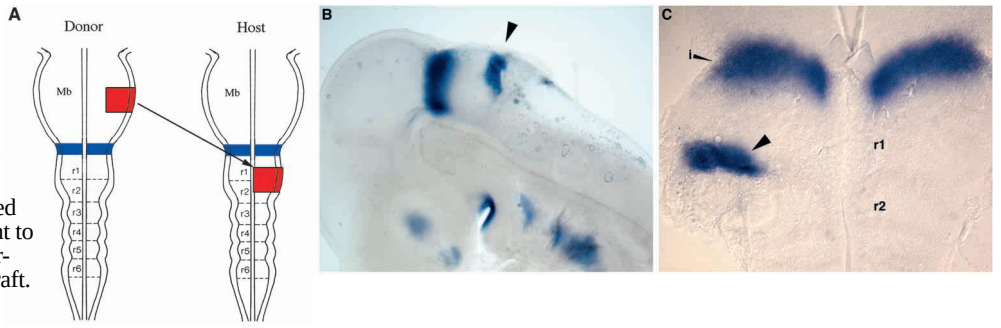

Fig. 4. *Fgf8* is induced when midbrain is grafted into posterior r1. Midbrain grafts (red) were made into posterior r1 and r2 as depicted in the diagram (A). (B,C) Ectopic expression of *Fgf8* was observed at the graft-host interface along the anterior edge of the graft (indicated by the arrowhead in B and C; C is a flat-mounted hindbrain). *Fgf8* was only induced where midbrain tissue was grafted adjacent to r1 tissue and never at the midbrain-r2 interface, regardless of the orientation of the graft. r1, rhombomere 1; r2, rhombomere 2; i, isthmus.

co-cultures of either r2 (*n*=0/2; data not shown) or r3 (*n*=0/6; Fig. 5G,H) with midbrain tissue. These data suggest that the signalling event responsible for the induction of *Fgf8* is mediated via a diffusible molecule.

Origin of regenerated isthmic cells

Fgf-8-positive isthmic tissue is normally located within the anterior limit of the *Gbx2* expression domain and adjacent to the posterior limit of *Otx2* transcripts (Shamim and Mason, 1998; Shamim et al., 1999). Therefore, it might be expected that all of the *Fgf8*-expressing cells induced in our grafting and explant studies might originate from the r1 tissue (i.e. from *Gbx2*-positive cells). Consistent with this, our transfilter experiments indicated that *Fgf8* was induced predominantly in the r1 tissue. We therefore sought to address the origin of the *Fgf8*-positive cells *in vivo* by using chick-quail chimeras in order to distinguish between r1 and midbrain tissue using a quail-specific antibody. Quail donor r1 tissue was grafted into chick host midbrain (Fig. 6A) and embryos were analysed for *Fgf8* induction. Grafted quail r1 tissue was found to be double-labelled for *Fgf8* mRNA and QCPN antigen (n=14/23; Fig. 6B-E and Table 1) with highest apparent levels of *Fgf8* transcripts associated with one edge of the graft. Elevated expression along one edge of the graft may reflect a preference for the most anterior r1 tissue in the graft to form new isthmic tissue. To test this, the anterior edge of the r1 graft was labelled with DiI prior to its excision from the donor brain. Cells labelled with DiI subsequently appeared associated with the domains of highest expression of *Fgf8* suggesting that induction occurs preferentially in the more anterior part of the r1 graft. This apparent polarity of response in the r1 graft was observed regardless of the orientation of the grafted tissue with respect to the host midbrain (Fig. 6B,D). Grafts of quail midbrain into host r1 likewise showed that *Fgf8* was predominantly induced in r1 tissue (Fig. 6F-H) but induction occurred irrespective of the orientation of the midbrain graft (*n*=7/8 for normal polarity, *n*=3/4 for reversed polarity).

In some cases, induced *Fgf8* expression was restricted to the r1 tissue, although in other instances a small number of positive cells were clearly derived from midbrain tissue (Fig. 6E,F). But it should be noted that, in all cases, the majority of *Fgf8*-positive cells were of r1 origin. These observations prompted us to investigate whether expressing cells of midbrain origin might be due to the re-specification of midbrain tissue adjacent

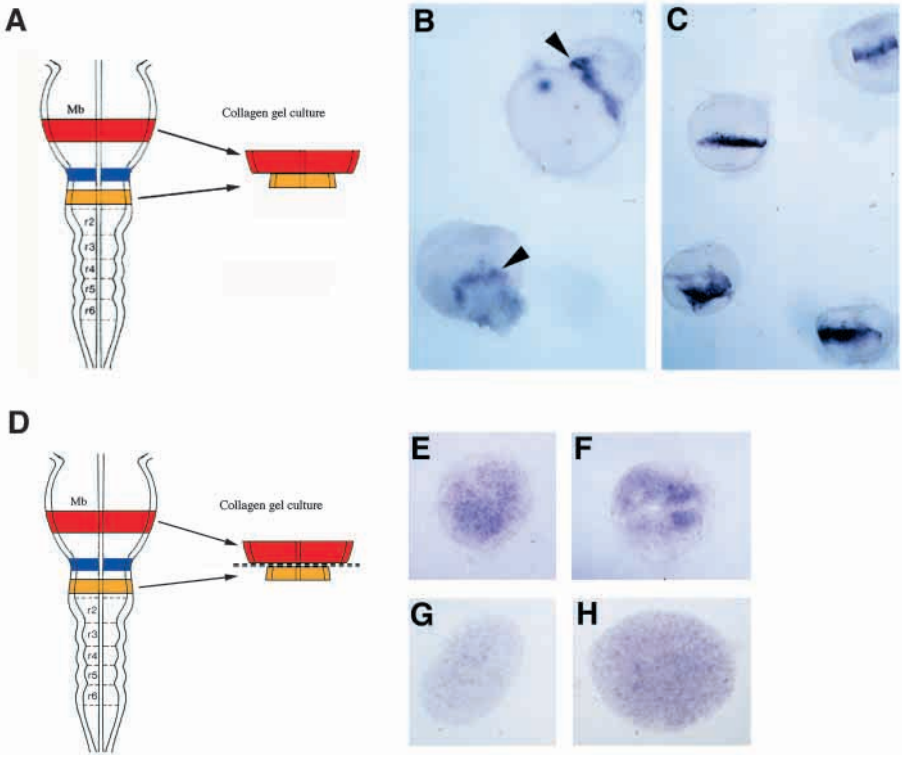

Fig. 5. *Fgf8* is induced in co-cultured explants of midbrain and r1. (A-C) Explants of anterior midbrain (red) and posterior r1 (yellow) were excised and cultured in a collagen gel matrix as shown in A. (B) In situ hybridisation to *Fgf8* revealed that transcripts were induced within the explant when midbrain and r1 were juxtaposed (indicated by the arrowhead). (C) The control isthmic region between the explants maintained a sharp band of *Fgf8* expression in culture, and demonstrates a clear *Fgf8*-negative territory either side of this. (D-H) *Fgf8* is induced when anterior midbrain and posterior r1 are separated in trans-filter collagen co-culture. (D) A filter of varying pore size was placed between the midbrain and r1 tissues as depicted. (E,F) Induction of *Fgf8* in r1 was observed by in situ hybridisation when explants were separated from midbrain by a 0.4 μm filter which prevented cell contact-dependent signalling but still allowed the passage of diffusible molecules. (G,H) *Fgf-8* is not induced in r3 in similar transfilter cultures with midbrain.

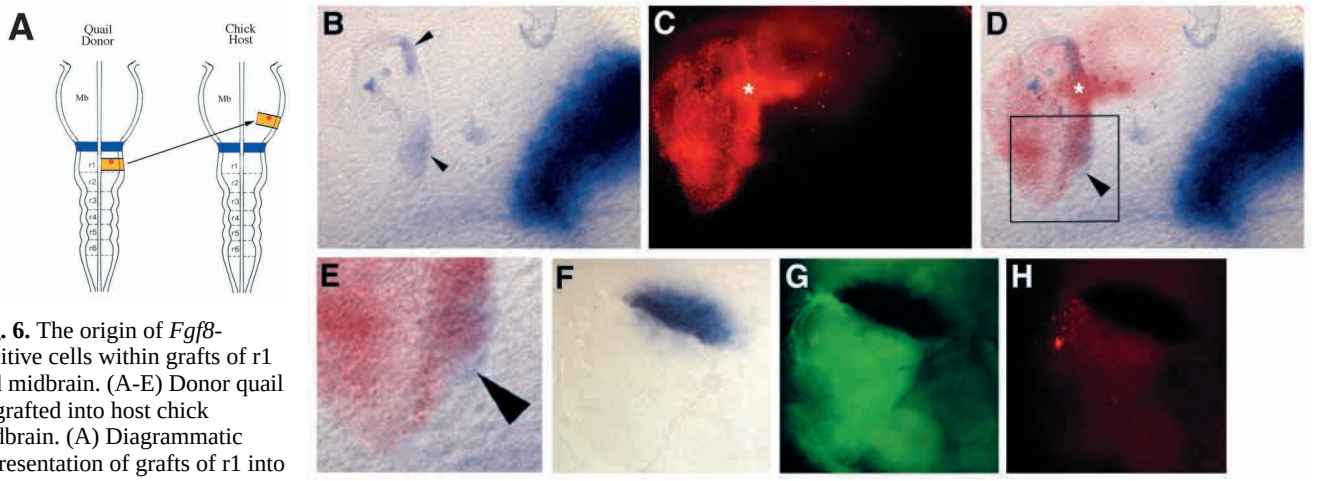

Fig. 6. The origin of *Fgf8*-positive cells within grafts of r1 and midbrain. (A-E) Donor quail r1 grafted into host chick midbrain. (A) Diagrammatic representation of grafts of r1 into midbrain: Quail posterior r1 (labelled with DiI at the anterior edge; red dot) was grafted heterotopically into chick host midbrain at HH stage 10. (B-E) Graft of r1 into midbrain in which the graft was inserted with reversed A-P polarity. (B) Ectopic *Fgf8* expression is detected within the r1 graft (arrowheads) as indicated by immunohistochemical detection of quail tissue (C). *Star in C indicates the position of the DiI label marking the anterior of the graft. (D) Ectopic expression of *Fgf8* detected by in situ hybridisation is indicated by the arrowhead at the graft-host interface in a combined image of *Fgf8* expression (B) and QCPN staining (C) to detect quail tissue. The star indicates the site of DiI injection that marks the anterior edge of the r1 graft. (E) Increased magnification of the boxed area in D shows that some *Fgf8*-postive cells are derived from chick host midbrain in this example (arrowhead). (F-H) Grafts of quail midbrain tissue into posterior host r1 in a manner identical to that shown in Fig. 4A but with the anterior of the graft labelled with DiI. (F) Ectopic *Fgf8* transcripts are detected at the midbrain-r1 interface and are largely confined to r1 tissue but note some postive cells within the grafted midbrain. (G) Detection of quail tissue with QCPN antibody. (H) Detection of the DiI label marking the anterior region of the graft.

to the graft to an anterior hindbrain phenotype (i.e. *Gbx2*+, *Otx2*–). Grafts of quail r1 into chick midbrain were assayed for expression of *Gbx2*. We found that expression was maintained within the graft but in some cases it was also detected in midbrain cells adjacent to it (*n*=5/11; Fig. 7A-C and Table 1). Our previous studies have shown that FGF8-soaked beads can induce *Fgf8* expression in midbrain tissue (Shamim et al., 1999). We therefore investigated whether Fgf8, induced in the r1 explant, might be capable of inducing *Gbx2* observed in adjacent midbrain cells. FGF8-coated beads were implanted into midbrains and embryos assayed 24 hours later for *Gbx2* transcripts. We found a small domain of *Gbx2*-positive cells induced adjacent to the bead (*n*=3/4; Fig. 7D). This suggests that (i) the *Fgf8* transcripts sometimes observed in a few midbrain cells adjacent to the r1 graft arise from local respecification of midbrain to an anterior hindbrain character and (ii) that this is probably mediated by Fgf8 induced initially in the r1 graft.

Evidence that a functional isthmus is generated following juxtaposition of midbrain and r1

A characteristic early response to grafts of isthmic tissue into midbrain is ectopic expression of *Wnt1* which is induced both around the graft and extending to the graft from the normal domain of expression in the dorsal midline (Bally-Cuif and Wassef, 1994). We were therefore interested to see if the *Fgf8*-positive 'isthmic' tissue generated in r1 grafted into midbrain, shared this property. In the majority of cases (n=8/10; Table 1), *Wnt1* transcripts were observed ectopically within the midbrain on the side of the embryo that received the graft. Two patterns of ectopic expression were observed. In some cases, a stripe of *Wnt1*-expressing cells projected from the dorsal midline and extended down adjacent to the r1 graft in a similar manner to that reported for grafts of isthmic tissue (Fig. 8A). In other cases, there was simply a broadening of the dorsal midline expression towards the most dorsal part of the r1 graft (Fig. 8B-D). *Wnt1* expression was never seen within the grafted quail r1 tissue.

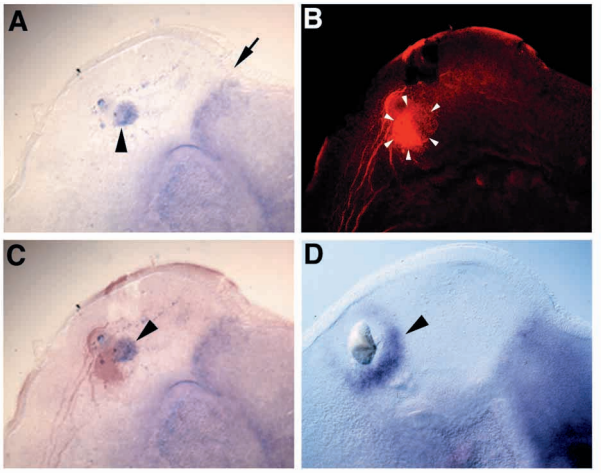

Fig. 7. Re-specification of host midbrain cells adjacent to the r1 graft. (A) In situ hybridisation to detect *Gbx2* expression following grafting of a quail posterior r1 into chick midbrain. The arrow indicates the normal boundary of *Gbx2* expression at the midbrain-hindbrain junction. The arrowhead indicates ectopic expression of *Gbx2* associated with the graft. (B) Donor quail r1 cells identified by the QCPN antibody. The arrowheads indicate the limits of the graft. (C) In a combined image of A and B some cells derived from the chick midbrain (QCPN-negative) are *Gbx2*-positive (indicated by the arrowhead). (D) *Gbx2* expression is induced in the midbrain by an FGF8-soaked bead. The arrowhead indicates ectopic expression around the bead.

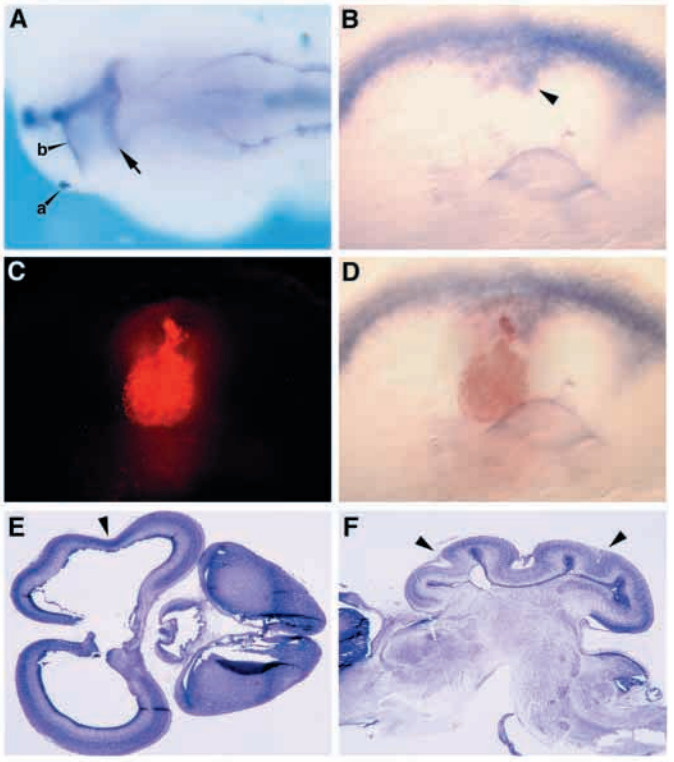

Fig. 8. A functional isthmus is formed following grafts of r1 into midbrain. (A) In situ hybridisation to *Wnt1* following a graft of quail posterior r1 into chick midbrain. Ectopic *Wnt1* expressing cells are induced around the r1 graft. The embryo is seen from a dorsal view; the graft is placed on the left side of the midbrain. Ectopic *Wnt1* cells were observed adjacent to the graft (a) and projecting from the dorsal midline towards the graft (b). Normal expression at the isthmus is indicated by the arrow. (B) Lateral view of a midbrain showing an ectopic broadening of the dorsal midline expression towards the dorsal part of the graft (arrowhead). (C) Grafted tissue in B was detected by the QCPN antibody. (D) The width of the ectopic expression in B correlated with the width of the graft when the images in B and C were overlaid. (E) Coronal section taken through the tectum and telencephalon of an embryo allowed to develop for 8 days (E10.5) following a graft of r1 into midbrain. Arrowhead indicates a thinning of the tectal wall associated with regions of opposing polarity. (F) Sagittal section of an embryo following a graft of r1 into midbrain allowed to develop for 8 days in which a foliated and thickened tectal morphology is observed (arrowheads).

To further examine whether or not a functional isthmic organiser is induced following interactions between midbrain and r1, embryos in which r1 had been grafted into midbrain were allowed to develop for between 7 and 8 days. Following sectioning and staining with cresyl violet, we observed changes in tectal morphology consistent with induction of an ectopic isthmic organiser (n=4/7; Fig. 8E). Alterations in tectal polarity were similar to those previously reported following both grafts of isthmic tissue and implantation of FGF8-soaked beads into midbrain (Marin and Puelles, 1994; Shamim et al., 1999; Martinez et al., 1999). In addition, one embryo was found to have a foliated tectal morphology (Fig. 8F) which we have also recently reported as an alternative morphology seen following FGF8 bead implants (Shamim et al., 1999).

DISCUSSION

The experiments described above concern the regeneration and reformation of isthmic tissue following ablation, tissue grafting in ovo or tissue recombinations in vitro. These data provide insights into the mechanisms which maintain isthmic function during development and may also prove informative concerning initiation of isthmus formation.

Previous studies within the hindbrain have shown that inter-rhombomeric boundaries reform following aspiration (Guthrie and Lumsden, 1991; Mahmood et al., 1995b). Our observations of the midbrain-hindbrain boundary reveal a robust ability to regenerate after removal of all boundary cells suggesting that, in accordance with the hindbrain, the isthmus is maintained and/or generated by interactions between cells in adjacent compartments.

Grafting studies of individual rhombomeres into midbrain, midbrain tissue into hindbrain and co-cultures of rhombomeres with midbrain tissue in vitro, showed that *Fgf8* expression was only generated when r1 tissue was juxtaposed to midbrain. Thus, induction of *Fgf8* expression is not a general property of hindbrain tissue and does not follow the odd/even rule identified for inter-rhombomeric boundaries (Guthrie and Lumsden, 1991; Mahmood et al., 1995b). Moreover, it is unlikely that *Gbx2* directly determines the ability of r1 to respond since, at the time of grafting, r2 and possibly r3, also express *Gbx2* (Shamim and Mason, 1998), and mice lacking *Gbx2* function still express *Fgf8* (Wassarmann et al., 1997).

When posterior r1 was grafted into the anterior midbrain, *Fgf8* was induced with highest levels along one edge of the donor-host interface. Using DiI to mark the most anterior part of the r1 graft, we found that the region expressing the highest apparent levels of *Fgf8* mRNA was associated with the anterior of the graft regardless of its orientation in the host midbrain. Similar studies of midbrain tissue when grafted into hindbrain further indicated a lack of polarity in the former tissue in its ability to induce *Fgf8*. The polarity of the r1 response correlates with A-P gradients of expression of a number of markers within avian r1, including *En1, En2, Pax2, ephrin A2* and *ephrin A5* (reviewed by Joyner, 1996; C. I. and I. M., unpublished data; A. Gustafson and I. M., unpublished observations). However, while these provide further evidence of an intrinsic polarity within r1, they are also expressed within the midbrain and therefore are unlikely to participate in the induction of *Fgf8* observed in our studies. It should also be noted that, while there is a clear preference for new isthmic tissue to be generated in the anterior of r1, grafts of midbrain adjacent to posterior r1 indicate that even the most posterior r1 tissue is competent to respond (see Fig. 4C).

The ectopic appearance of *Wnt1*-expressing cells when r1 is juxtaposed with midbrain complements observations following isthmus grafts into the midbrain (Bally-Cuif and Wassef, 1994). Moreover, the tectal morphology observed following incubation of embryos to later stages is identical to that observed following either grafts of isthmic tissue or implants of FGF8 beads into midbrain (Marin and Puelles, 1994; Shamim et al., 1999; Martinez et al., 1999). These data support the conclusion that an ectopic isthmus is induced in our studies which has organising activity characteristic of the normal isthmus. Theoretical considerations led Meinhardt to postulate a general mechanism for formation of boundaries separating

distinct cell populations and the subsequent organisation of new positional information around them (Meinhardt, 1983). This principle would explain both the formation of a new isthmus when r1 and midbrain are juxtaposed and the re-organisation of *Wnt1* around this tissue.

It is now clear from both lineage-tracing studies and from the use of molecular markers that the position of the morphological isthmic constriction moves anteriorly between HH stage 10 and HH stage 17 (Millet et al., 1996; Shamim et al., 1999). Taken together these studies show that while the constriction lies centrally within the *Fgf8*+ region of the isthmic organiser at HH stage 10, by HH stage 17 the *Fgf8*+ region lies immediately posterior to the constriction. However, the relative spatial relationships of gene expression (e.g. *Fgf8*, *Otx2*, *Gbx2* and *Wnt1*) and their relationships to cell fate remain unaltered. Thus, *Fgf8* expression is always located at and within the anterior of the *Gbx2*-positive domain and immediately posterior and adjacent to the *Otx2*-positive region. We found that most induced *Fgf8* transcripts were located within the r1 tissue, but that in some cases, expression was also detected in a few midbrain cells adjacent to the graft. However, further studies showed that the presence of the r1 graft caused a small region of midbrain tissue that was closely associated with it to express *Gbx2*. Moreover, FGF8 protein applied on beads could also produce this effect and others have shown that ectopic FGF8 represses *Otx2* expression within the midbrain (Martinez et al., 1999). These data would suggest that interactions between r1 and midbrain can induce *Fgf8* expression in r1 and that FGF8 can then cause midbrain cells adjacent to the graft to express both *Gbx2* and *Fgf8*. This raises the question as to why this process does not continue resulting in *Gbx2* and *Fgf8* expression spreading across the midbrain both during normal development and following grafts of r1 or FGF8-coated beads into midbrain. A candidate for an antagonist of this process is *Wnt1* which is normally expressed immediately anterior to the *Fgf8*-positive domain and is induced following grafting.

Co-culture of isolated pieces of midbrain and r1 tissue within a collagen matrix revealed that the inductive interaction leading to *Fgf8* expression is a direct effect, resulting from planar signalling between the two adjacent cell populations and not an indirect process involving other surrounding tissues. Furthermore, transfilter experiments revealed that this signalling event does not require direct cell contact between the two cell populations. These data strongly suggest that the induction of *Fgf8* is mediated by a diffusible signalling molecule. The identity of this molecule is currently unknown. It is unlikely that the signal is Wnt1 because, at the time of grafting, *Wnt1* is expressed in a broad domain throughout the midbrain, across the isthmus and within r1 (Shamim et al., 1999). Eph receptors and their ligands, the ephrins, provide good candidate regulators of boundary formation within the hindbrain (see Introduction). However they seem unlikely to be involved in the process of *Fgf8* induction as there are currently no r1-specific Eph proteins (all of those present in r1 are expressed elsewhere in the hindbrain). Furthermore, ephrins are not known to be diffusible and those that are expressed in the midbrain are also expressed in r1 (A. Gustafson and I. M., unpublished observations). Experiments are currently in progress to determine the identity of the signal and its receptor.

We thank Jonathan Slack for advice concerning the transfilter experiments, David Chambers for a valuable suggestion and Richard Wingate for discussion and advice about tissue grafts. Members of the Mason laboratory are thanked for their comments on the manuscript. This work was supported by the MRC, Wellcome Trust and Human Frontier Science Program.

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