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Roles of Eph receptors and ephrins in segmental patterning

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Eph receptor tyrosine kinases and their membrane-bound ligands, ephrins, have key roles in patterning and morphogenesis. Interactions between these molecules are promiscuous, but largely fall into two groups: EphA receptors bind to glycosylphosphatidyl inositol-anchored ephrin-A ligands, and EphB receptors bind to transmembrane ephrin-B proteins. Ephrin-B proteins transduce signals, such that bidirectional signalling can occur upon interaction with the Eph receptor. In many tissues, there are complementary and overlapping expression domains of interacting Eph receptors and ephrins. An important role of Eph receptors and ephrins is to mediate cell contact-dependent repulsion, and this has been implicated in the pathfinding of axons and neural crest cells, and the restriction of cell intermingling between hindbrain segments. Studies in an *in vitro* system show that bidirectional activation is required to prevent intermingling between cell populations, whereas unidirectional activation can restrict cell communication via gap junctions. Recent work indicates that Eph receptors can also upregulate cell adhesion, but the biochemical basis of repulsion versus adhesion responses is unclear. Eph receptors and ephrins have thus emerged as key regulators that, in parallel with cell adhesion molecules, underlie the establishment and maintenance of patterns of cellular organization.

Keywords: segmentation; cell signalling; receptor tyrosine kinase; cell movement

1. INTRODUCTION

One major aim of developmental biology is to identify the mechanisms that generate specific organized patterns of distinct cell types during embryogenesis. There has been much progress in the identification and analysis of intercellular signals and transcription factors involved in the induction of specific tissues or cell types at appropriate locations in the developing embryo. However, less is known regarding the mechanisms that control cell movements crucial for patterning and morphogenesis. For example, stereotyped movements such as convergent extension, and the migration of mesenchymal cells to specific destinations, are crucial for the morphogenesis and patterning of a number of tissues. In addition, there can be much movement and dispersal of clonally related cells, due to repeated rounds of division and the intercalation of adjacent cells (see, for example, Kimmel *et al*. 1994). This raises the question as to how organized patterns are maintained despite such intermingling that has the potential to scramble distinct tissues, or domains within a tissue that will later form different derivatives. Similarly, how are patterns maintained in distinct populations of mesenchymal cells that have the potential to intermingle as cells migrate?

Two general mechanisms can be envisaged to underlie the maintenance of organized patterns despite cell intermingling. One mechanism involves a plasticity of cell specification, and local signals that cause any cells that cross into an adjacent territory to switch to the same identity as their new neighbours. The other involves a specific restriction of cell movement between adjacent cell populations. There is good evidence for each of these mechanisms, which may act alone, or in parallel, to stabilize patterns and maintain sharp interfaces between distinct cell populations.

There is much evidence for a key role of cell adhesion molecules in stabilizing tissues by the establishment of differences in cell–cell affinity. Classical experiments have shown that when tissues are dissociated, mixed and reaggregated *in vitro*, cells from different tissues sort out to form segregated cell populations (Townes & Holfreter 1955). This cell sorting can be explained by a model in which during intermingling, cells of the same type preferentially associate because they have a stronger affinity for each other than they do for a different cell type (Steinberg 1970). Similar *in vitro* cell sorting occurs between cells expressing distinct cell adhesion molecules, or different levels of the same cell adhesion molecule (Nose *et al*. 1988; Friedlander *et al*. 1989). Taken together with the effect of null mutations, and of blocking or ectopic expression of cell adhesion molecules *in vivo* (e.g. Bradley *et al*. 1998; Godt & Tepass 1998; Gonzalez-Reyes & St Johnston 1998), these findings reveal a crucial role of tissue-restricted cell adhesion molecules in stabilizing patterns of cellular organization (reviewed by Takeichi 1991; Gumbiner 1996). Recent work has shown that another class of molecules—Eph receptors and their ephrin ligands—also contribute to the stabilization of tissue patterns. This review will focus on the roles of Eph receptors and ephrins in segmental patterning, and highlight the conclusions and questions raised by these and other studies of their functions in morphogenesis.

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2. EPH RECEPTORS AND EPHRINS

In vertebrates, Eph receptors comprise a family of 14 receptor tyrosine kinases that interact with a family of eight membrane-bound ephrin ligands (Eph Nomenclature Committee 1997). Recently, an Eph receptor gene has been found in *Caenorhabditis elegans* (George *et al*. 1998), *Drosophila* (Scully *et al*. 1999) and sponges (Suga *et al*. 1999), suggesting that they have an ancient role in multicellular animals. The most distinctive feature of Eph receptors is the primary structure of the extracellular region, which includes two fibronectin type III motifs (Pasquale 1991), 20 conserved cysteines, many of which are clustered in a cysteine-rich region, and an N-terminal ligand-binding domain (Labrador *et al*. 1997; Lackmann *et al*. 1998). Based on amino-acid sequence similarities (see Gale *et al*. 1996*a*), vertebrate Eph receptors can be divided into two subclasses, EphA (EphA1 to EphA8) and EphB (EphB1 to EphB6). Ephrins fall into two structural classes, with the ephrin-A proteins (ephrin-A1 to ephrin-A5) anchored in the plasma membrane through a glycosylphosphatidyl inositol linkage, whereas ephrin-B proteins (ephrin-B1 to ephrin-B3) have a transmembrane region and short cytoplasmic region. At the C-terminal end of this cytoplasmic region are 33 highly conserved amino acids including five tyrosine residues. Interactions between Eph receptors and ephrins largely fall into two binding-specificity classes. EphA receptors bind the ephrin-A ligands, whereas EphB receptors bind the ephrin-B proteins; an exception is the EphA4 receptor that binds ephrin-B2 and ephrin-B3 as well as ephrin-A ligands (Gale *et al*. 1996*a*).

Membrane-bound ephrins trigger Eph receptor phosphorylation, whereas soluble forms bind to Eph receptor but do not trigger receptor activation (Davis *et al*. 1994). However, soluble ephrins activate the receptor when they are artificially aggregated (Davis *et al*. 1994), and there is evidence that higher-order clusters may stimulate distinct responses from dimers (Gale & Yancopoulos 1997; Stein *et al*. 1998). These findings show that Eph receptors and ephrins mediate contact-dependent cell interactions, and suggest that membrane anchoring of ephrins may enable their clustering before or upon binding to Eph receptor.

The strong amino-acid sequence conservation in the intracellular domain of ephrin-B family members raised the possibility that these proteins may themselves transduce signals, and this received indirect support from analysis of mutants of the *EphB2* gene (Henkemeyer *et al*. 1996). Biochemical evidence was obtained in experiments showing that tyrosine phosphorylation of ephrin-B1–B2 protein occurs upon interaction with clustered soluble or membrane-bound EphB2, presumably by recruitment of a cytoplasmic kinase to the ephrin-B cytoplasmic domain (Holland *et al*. 1996; Bruckner *et al*. 1997). Thus, interaction between cells expressing Eph receptor with cells expressing ephrin-B may lead to bidirectional signal transduction, with each component acting as both 'receptor' and 'ligand'.

Gene-expression studies have shown that, collectively, the *Eph* receptor and *ephrin* gene families are expressed in complex patterns in many, perhaps all tissues throughout development and in the adult (for references, see Flanagan & Vanderhaeghen 1998; Wilkinson 2000). Individual members of the same *Eph* receptor or *ephrin* class can have the same as well as distinct sites of expression, raising the possibility that family members could have overlapping or synergistic roles in some tissues. Several examples have been found in which, in different species, a different *Eph* receptor or *ephrin* is expressed in a specific tissue (Wang & Anderson 1997; Feldheim *et al*. 1998), suggesting that some members of the same class may be functionally interchangeable and have similar or identical biochemical properties. Importantly, expression studies have shown that interacting Eph receptors and ephrins are in some regions expressed in complementary domains, whereas in other regions there are overlaps (e.g. Flenniken *et al*. 1996; Gale *et al*. 1996*a*; Connor *et al*. 1998; Adams *et al*. 1999; Sobieszczuk & Wilkinson 1999). There have been major advances in understanding developmental roles of complementary Eph receptor and ephrin expression, and recent work has started to elucidate the significance of overlapping expression.

3. ROLES IN AXONAL PATHFINDING

There is now much evidence that Eph receptors and ephrins have key roles in guiding neuronal growth cones (reviewed by Drescher *et al*. 1997; Orioli & Klein 1997; Flanagan & Vanderhaeghen 1998; O'Leary & Wilkinson 1999). In the retinotectal system and other topographic maps, gradients of an EphA receptor in neurons and of ephrin-A ligands in the target tissue underlie a graded repulsion of growth cones that establishes a spatial mapping of projections (Drescher *et al*. 1995; Nakamoto *et al*. 1996; Monschau *et al*. 1997; Zhou 1997; Feldheim *et al*. 1998; Frisen *et al*. 1998). Eph receptors and ephrins can also act as repellents at boundaries to prevent axons from entering specific territories, and thus channel them towards their targets (Henkemeyer *et al*. 1996; Orioli *et al*. 1996; Wang & Anderson 1997; Dottori *et al*. 1998). Studies of growth cone collapse responses to ephrin repellents (Meima *et al*. 1997*a*,*b*), and of the biochemical pathways triggered by Eph receptor activation (reviewed by Bruckner & Klein 1998), suggest that the actin cytoskeleton is a major target of signalling. It is therefore believed that the complementary expression of Eph receptors and ephrins may have a general role in preventing neuronal growth cones from entering inappropriate territories. As will be discussed below, there is a strong parallel between roles in axonal pathfinding and at earlier stages of patterning.

4. RESTRICTION OF CELL INTERMINGLING DURING HINDBRAIN SEGMENTATION

The hindbrain is subdivided into repeated morphological units, termed rhombomeres, that underlie a segmental organization of nerves and of neural crest cells that migrate in streams into the branchial arches. These cellular patterns are established by the segmental expression of genes such as *Krox-20* required for the formation of segments, and by *Hox* genes that confer anteroposterior (A–P) identity (reviewed by McGinnis & Krumlauf 1992; Wilkinson 1993; Lumsden & Krumlauf 1996). The expression domains of these segmentation and segment

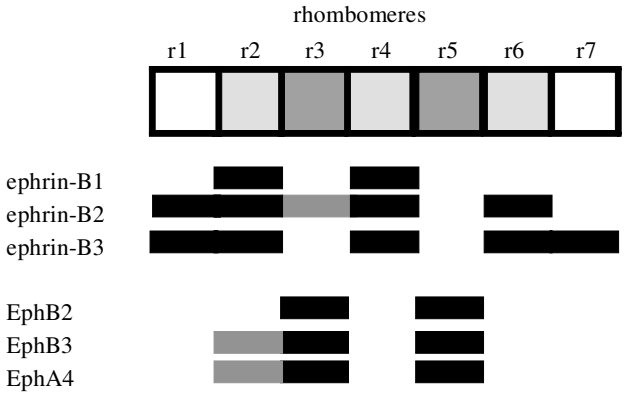

Figure 1. Expression patterns of Eph receptors and ephrins in the developing hindbrain. The diagram illustrates the expression domains in the hindbrain of ephrin-B proteins and Eph receptors that they interact with. There is both complementarity and overlap between the expression domains of these ephrins and Eph receptors. The EphA2 and EphA7 receptors are also expressed in the hindbrain (not shown) but ephrin-A ligands that interact with these have not been detected in the hindbrain.

identity genes have sharp boundaries, which are likely to underlie a homogeneous specification of segments that establishes precise patterns of neuronal organization. Hindbrain patterning thus provides an example of an important general question: What are the mechanisms that establish and maintain precise patterns of gene expression and tissue organization?

Studies of cell lineage have shown that whereas there is substantial cell intermingling between presumptive rhombomeres, after morphological segmentation there is a major restriction to cell movement between adjacent segments (Fraser *et al*. 1990). Taken together with studies of segmental gene expression, these findings suggest that a local regulation of cell identity and the segmental restriction of cell movement may both contribute to the maintenance and sharpening of segmental domains (Irving *et al*. 1996). The restriction of cell movement between adjacent segments is due to a cellular property that is present in alternating rhombomeres, such that r2/r4/r6 can intermingle with each other, and so can r3/r5, but cells from even-numbered segments do not intermingle with cells from odd-numbered segments (Guthrie *et al*. 1993).

One potential mechanism for restricting intermingling between rhombomeres is that a cell adhesion molecule(s) underlies a differential adhesion of cells in odd- versus even-numbered rhombomeres (Wizenmann & Lumsden 1997), but an adhesion protein with alternating segmental expression has not been discovered. The expression patterns of Eph receptors and ephrins are consistent with the possibility that they restrict cell movements between hindbrain segments. *EphA4*, *EphB2* and *EphB3* are expressed at high levels in rhombomeres r3/r5 (Nieto *et al*. 1992; Becker *et al*. 1994; Henkemeyer *et al*. 1994), whereas *ephrin-B1*, *ephrin-B2*, and *ephrin-B3* are expressed at high levels in r2/r4/r6 (Bergemann *et al*. 1995; Flenniken *et al*. 1996; Gale *et al*. 1996*b*) (figure 1). Due to this complementary expression, interactions of EphA4 and EphB receptors with ephrin-B proteins will occur at the interface of adjacent rhombomeres. However, there are also some overlaps in expression of Eph receptors and ephrins, at least in r2 and r3 (figure 1).

5. CELLULAR RESPONSES REGULATED BY EPH RECEPTORS AND EPHRINS IN THE HINDBRAIN

We obtained initial clues to roles of Eph receptors in the hindbrain in experiments in which truncated EphA4 lacking the kinase domain was expressed widely in zebrafish embryos by RNA injection at the one- or two-cell stage (Xu *et al*. 1995). Due to the phenomenon of bidirectional activation, truncated EphA4 may act in a dominant negative manner to block endogenous Eph receptors, and as a ligand that ectopically activates ephrin-B proteins. In contrast to control uninjected embryos (figure 2*a*), cells with r3/r5 identity were often present in r2/r4/r6, sometimes causing a fusion of r3 and r5 territories (figure 2*c*). Similar results were obtained when exogenous ephrin-B2 was widely expressed in zebrafish embryos, such that EphA4 and EphB receptors would be activated throughout r3/r5, rather than directionally at rhombomere boundaries (figure 2*b*). These phenotypes are consistent with several possible models. Blocking or activation of Eph receptors or ephrins could cause some cells with r2/r4/r6 identity to switch to r3/r5 identity, or could block normal switches in identity that occur when cells intermingle between presumptive odd and even segments. Alternatively, there could be a disruption of the normal restriction of intermingling between odd and even segments.

To distinguish between these possibilities, we took advantage of the extensive mixing of cells during early zebrafish development, such that when one cell is injected with *lacZ* RNA at the eight-cell stage, its descendants have a scattered distribution at neurula stages (figure 2*d*). By co-injecting *lacZ* and *ephrin-B2* RNA, we could ask whether mosaic activation of EphA4 and EphB receptors by this ephrin leads to changes in the identity or movement of cells within r3/r5 (Xu *et al*. 1999). Cells expressing *ephrin-B2* were found to become restricted to the boundaries of r3/r5, whereas in r2/r4/r6 expressing cells are scattered throughout the segment (figure 2*e*). The expression patterns of markers of r3/r5 identity are not altered, indicating that the mosaic expression of *ephrin-B2* does not alter the identity of the expressing or adjacent cells. Similar cell sorting was observed after mosaic expression of truncated ephrin-B2 (lacking the intracellular domain) that can activate Eph receptors, but cannot itself transduce a signal (Xu *et al*. 1999). Thus, mosaic activation of Eph receptors is sufficient for cell sorting. By analogy with the effects of differential cell adhesion (Steinberg 1970), sorting could be explained by a cell repulsion response to Eph receptor activation that leads to an affinity difference between r3/r5 cells expressing exogenous ephrin-B2 and those that are not. Consistent with a repulsion or de-adhesion response, there are larger intercellular spaces at rhombomere boundaries (Lumsden & Keynes 1989; Heyman *et al*. 1993) where Eph receptor–ephrin-B interactions are occurring.

In view of evidence that ephrin-B proteins may transduce signals, we analysed the effect of activating these

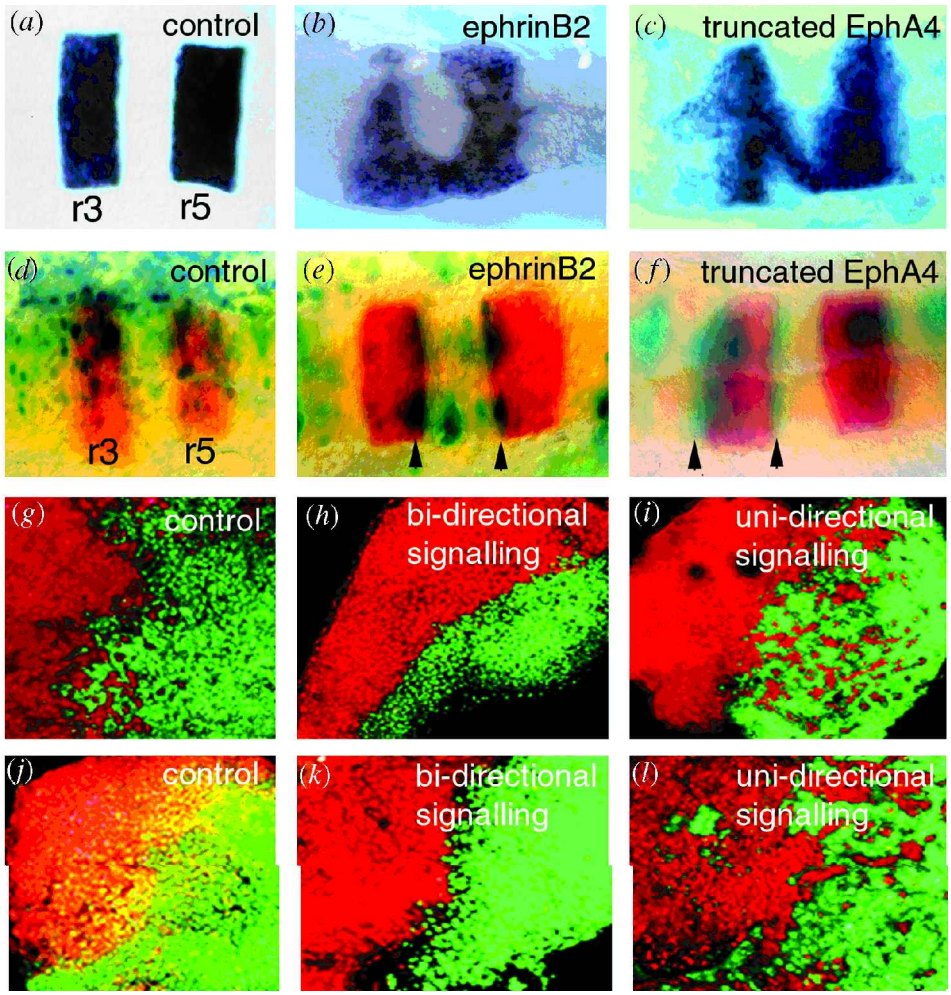

Figure 2. Roles of Eph receptors and ephrins in the control of cell movement. The panels summarize the results of different approaches to investigate responses to Eph receptor and ephrin signalling in zebrafish hindbrain patterning and in an *in vitro* system. (*a–c*) Effects of widespread blocking or ectopic activation of Eph receptors. The indicated proteins were expressed by RNA injection at the one- to two-cell stage *in vivo*. (*a*) Control uninjected embryo showing sharply restricted r3/r5 domains marked by Krox-20 gene expression (blue stain). (*b*) After widespread expression of ephrin-B2 to ectopically activate Eph receptors there are ectopic r3/r5 cells and often a fusion of these segments. (*c*) A similar phenotype is observed after widespread expression of truncated EphA4 that will block Eph receptor activation, and activate ephrin-B proteins. (*d–f*) Effects of mosaic activation of Eph receptors or ephrin-B proteins *in vivo*. The indicated proteins were coexpressed with β-galactosidase in a mosaic fashion by RNA injection into one cell at the eight-cell stage. The distribution of β-galactosidase (blue stain) and of Krox-20 as a marker of r3/r5 (red stain) was visualized. (*d*) Control injection of only lacZ RNA showing mosaic distribution due to intermingling during early development. (*e*) If RNA encoding ephrin-B2 is co-injected, the expressing cells sort to the boundaries of r3/r5 (arrowheads). (*f*) If RNA encoding truncated EphA4 is co-injected, the expressing cells sort to the boundaries of r2/r4/r6. (*g–i*) Fishball assays for cell intermingling *in vitro*. Zebrafish animal caps labelled with rhodamine dextran (red signal) or fluorescein dextran (green signal) were juxtaposed, cultured overnight and the distribution of cells visualized by confocal microscopy. (*g*) In a control assay with no co-injected reagents, cell intermingling occurs. (*h*) Expression of EphB2 receptor in one population and of ephrin-B2 in the other leads to bidirectional signalling that restricts cell intermingling. (*i*) Expression of truncated EphB2 in one population and of ephrin-B2 in the other leads to unidirectional signalling, but this does not restrict cell intermingling. (*j–l*) Fishball assays for gap junctional communication *in vitro*. Zebrafish animal caps labelled with rhodamine dextran (red) or Lucifer yellow (green signal) are juxtaposed and cultured overnight. Transfer of Lucifer yellow into rhodamine dextran-labelled cells via gap junctions is seen as a yellow signal. (*j*) In a control assay with no co-injected reagents gap junctional communication occurs. (*k*) Bidirectional activation of EphB2 and ephrin-B2 restricts gap junctional communication. (*l*) Unidirectional activation of ephrin-B2 by truncated EphB2 restricts gap junctional communication despite cell intermingling. Data in (*c*) from Xu *et al.* (1995); data in (*d–f*) from Xu *et al.* (1999); data in (*g–l*) from Mellitzer *et al.* (1999).

proteins in a mosaic fashion in the hindbrain. We visualized the distribution of cells expressing truncated EphA4 that can activate ephrin-B proteins, but cannot itself transduce a signal (Xu *et al.* 1999). Cells expressing truncated EphA4 were found to sort adjacent to the boundaries of r2/r4/r6 that express endogenous ephrin-B proteins, whereas labelled cells are frequently present in central regions of r3/r5 (figure 2*f*). One explanation is that ephrin-B activation can drive cell sorting via differences in cell–cell affinities, due to a repulsion or de-adhesion response similar to that occurring after Eph receptor activation.

These findings indicate that mosaic activation of Eph receptors or of ephrin-B proteins can each drive cell sorting, but it is not clear why the cells expressing ligand

(truncated receptor or ephrin) sort to rhombomere boundaries rather than within the segment. One possibility is that interactions of endogenous Eph receptors and ephrins at rhombomere boundaries create a zone with lower cell–cell affinities compared with non-boundary regions. Due to repulsive interactions, cells expressing ligand may have a similar lower affinity for their neighbours and thus sort preferentially to the boundaries.

6. REGULATION OF CELL INTERMINGLING AND COMMUNICATION BY EPH RECEPTORS AND EPHRINS

The finding that mosaic activation of Eph receptors or of ephrin-B proteins can drive cell sorting suggests that they may each trigger responses that affect cell affinities. This raises the question as to whether bidirectional activation at interfaces of Eph receptor–ephrin expression domains has an important role. To test this, we established and used an *in vitro* assay (Mellitzer *et al*. 1999). One-cell stage zebrafish embryos are injected with fluorescent lineage tracer and then animal caps dissected at the 1000-cell stage. After juxtaposing two animal caps, one labelled with rhodamine dextran and the other with fluorescein dextran, they rapidly adhere to form a fishball that is cultured overnight. Confocal microscopy reveals that intermingling occurs between control animal caps (figure 2*g*). In contrast when cells expressing ephrin-B2 are juxtaposed with cells expressing EphB2 and/or EphA4, there is a major restriction of intermingling between the cell populations (figure 2*h*). This restriction does not occur if Eph receptor or ephrin is omitted from one of the two cell populations, indicating that activation of any endogenous EphB receptors or ephrin-B proteins is not sufficient to restrict cell intermingling. To test whether the restriction of cell intermingling requires bidirectional activation, we carried out fishball assays in which there was unidirectional activation of EphA4 or EphB2 receptor by truncated ephrin-B2, or of ephrin-B2 by truncated EphB2. We found that after unidirectional signalling there is extensive intermingling between the two cell populations (figure 2*i*) (Mellitzer *et al*. 1999). A caveat is raised by the possibility that the intracellular domain of Eph receptor or of ephrin-B is required for them to be fully active as ligands, for example by mediating interactions with intracellular proteins that could cluster them (Hock *et al*. 1998; Torres *et al*. 1998; Bruckner *et al*. 1999; Buchert *et al*. 1999; Lin *et al*. 1999). To test this, we took advantage of the different binding specificities of Eph receptors and ephrins to reconstruct bidirectional signalling from unidirectional activation in each direction using truncated Eph receptor and ephrin as ligands. Cell intermingling was restricted in this situation (Mellitzer *et al*. 1999). Thus, bidirectional signalling between two cell populations restricts their intermingling, but unidirectional signalling does not.

A further mechanism that may stabilize patterns in the hindbrain is suggested by the observation that there is a disruption to cell communication via gap junctions across rhombomere boundaries (Martinez *et al*. 1992). Gap junctions form by assembly of connexin proteins into channels between cells that allow passage of < 1.2 kDa molecules (Bruzzone *et al*. 1996; Kumar & Gilula 1996), and can be detected by the ability of Lucifer yellow to diffuse through these channels. The developmental roles of gap junctional communication are currently unclear, but it is likely that by allowing cells to share low molecular weight secondary messengers they enable coordination of cell proliferation or differentiation. Thus, disruption to gap junctional communication may be essential for adjacent cell populations to acquire differences in fate or proliferation. It seemed possible that the larger intercellular spaces at rhombomere boundaries (Lumsden & Keynes 1989; Heyman *et al*. 1993) are due to cell repulsion mediated by Eph receptor–ephrin interactions, and that this prevents stable cell contacts required for gap junction assembly. We tested this in fishball assays in which one animal cap labelled with Lucifer yellow (green in the confocal image), is juxtaposed with another labelled with rhodamine dextran (red fluorescence) (Mellitzer *et al*. 1999). In control fishballs, Lucifer yellow transfers into rhodamine dextran-labelled cells (the overlap leading to a yellow signal), indicating that gap junctions have formed between the cell populations (figure 2*j*). However, when EphA4 or EphB2 were expressed in one animal cap and ephrin-B2 in the other, Lucifer yellow did not diffuse between the cell populations (figure 2*k*). Furthermore, gap junction formation was prevented by unidirectional activation of ephrin-B2 or of EphB2 by truncated ligand (figure 2*l*) (Mellitzer *et al*. 1999).

These results can be explained by a model in which the activation of Eph receptor or ephrin each triggers a repulsion or de-adhesion response. At the interface of cells expressing Eph receptor and cells expressing ephrin-B, bidirectional activation leads to a mutual repulsion that prevents the movement of each cell population into the other, and restricts gap junction formation. In the hindbrain, this coordinated restriction of cell intermingling and communication may be crucial for the stabilization of segmental patterns. In contrast, unidirectional signalling will repel one population, but the cells expressing truncated Eph receptor or ephrin are not repelled, and can invade adjacent territory, leading to intermingling. However, repulsion of only one of the two cell populations is sufficient to prevent stable cell–cell contacts required for gap junction assembly, leading to an uncoupling of restrictions to cell mixing and communication. Since truncated forms of Eph receptors exist due to alternative splicing (reviewed by Pasquale 1997) it is possible that unidirectional activation occurs *in vivo*. It will be interesting to examine whether this could prevent gap junctional communication between intermingled cell populations.

7. RELATIONSHIPS BETWEEN CELL MIXING AND IDENTITY IN THE HINDBRAIN

The work discussed above suggests that Eph receptors and ephrins are involved in restricting cell intermingling between hindbrain segments. In view of the possibility that such restrictions act in parallel with a plasticity and local regulation of segmental identity, it is important to consider why disruptions to r3/r5 organization are seen after widespread expression of truncated EphA4 (Xu *et al*. 1995). Ectopic cells with r3/r5 identity are never

found to be isolated within r2/r4/r6, but rather form coherent groups contiguous with r3/r5. After injection of RNA encoding truncated EphA4 into one cell at the eight-cell stage, r3/r5 were altered in shape in only 5% of the embryos, compared with > 50% after injection at the two-cell stage. These data are consistent with the blocking of EphA4 in an increasing proportion of r3/r5 cells causing a greater number to intermingle into r2/r4/r6. It can be envisaged that local community effects will switch isolated ectopic r3/r5 cells to an even-numbered identity, whereas larger groups of ectopic cells can maintain their identity. According to this view, Eph receptor–ephrin interactions may be required *in vivo* to prevent the intermingling of cells from being so excessive that identity switching mechanisms are not able to maintain sharp patterns. It will be important to test this model by transplanting groups of cells between rhombomeres, and analysing the relationship between cell intermingling and identity, for example using green fluorescent protein reporter genes to visualize cell identity in living embryos.

Since the restriction of cell intermingling between rhombomeres by Eph receptors and ephrins requires that they are segmentally expressed, it is important to understand how this expression is regulated. Currently, nothing is known regarding the regulation of ephrin-B gene expression, but *EphA4* gene expression has been shown to be under the direct control of the Krox-20 zinc finger transcription factor (Theil *et al*. 1998). In addition to being required for the formation of definitive r3/r5 (Schneider-Maunoury *et al*. 1993; Swiatek & Gridley 1993), Krox-20 regulates the expression of the *Hoxa2* and *Hoxb2* genes (Sham *et al*. 1993; Nonchev *et al*. 1996). There is thus a coupling between segmentation, A–P positional specification and the segmental restriction of cell movement, and this may be important for the maintenance of segmental domains with distinct identity. Furthermore, there is evidence that expression of *EphA7* in r3/r5 is downstream of *Hoxa2* (Taneja *et al*. 1996), and that of *EphA2* in r4 is downstream of *Hoxa1* and *Hoxb1* (Studer *et al*. 1998), indicating that there is also coupling at a different step of the regulatory hierarchy. However, the role of these Eph receptors in the hindbrain is currently unknown.

8. ROLES IN RESTRICTING NEURAL CREST CELL MIGRATION

The complex expression of Eph receptors and ephrins in most if not all regions of the developing embryo (Gale *et al*. 1996*a*) raises the question as to whether they have general roles in stabilizing patterns of tissue organization. Although little is currently known regarding their roles in many tissues, there is evidence that Eph receptors and ephrins are involved in restricting the movement of cells in the neural crest and during somite formation.

Neural crest cells arise by the delamination of cells from the dorsolateral edge of the neural epithelium, and migrate along a variety of pathways to specific destinations (Le Douarin 1982; Bronner-Fraser 1993). In chick and rodent embryos, trunk neural crest cells migrate through the anterior but not the posterior half of each somite (Rickmann *et al*. 1985; Bronner-Fraser 1986), and this segmental migration underlies formation of the repeated pattern of dorsal root and sympathetic ganglia (Kalcheim & Teillet 1989; Goldstein & Kalcheim 1991). If the orientation of somites is reversed along the A–P axis, there is a corresponding reversal of the pattern of migration of neural crest cells (Bronner-Fraser & Stern 1991). A similar restriction imposed by the somites also occurs for trunk motor axons (Keynes & Stern 1984). Somites therefore guide neural crest cells and motor axons, perhaps due to attractive cues within the anterior half of each somite and/or repulsive cues within the posterior half.

There is evidence implicating a number of molecules expressed in the posterior half of somites in the restriction of neural crest cells and/or motor axons, including a peanut lectin-binding glycoprotein, type IX collagen and F-spondin (Stern *et al*. 1986; Davies *et al*. 1990; Krull *et al*. 1995; Ring *et al*. 1996; Debby-Brafman *et al*. 1999). In addition to these factors, ephrin-B proteins (ephrin-B1 in the chick, ephrin-B2 in rodents) are expressed in the posterior half of somites, and *in vitro* stripe assays show that they repel trunk neural crest cells and motor axons that express EphB receptors (Krull *et al*. 1997; Wang & Anderson 1997). As observed in stripe assays of retinal axons (Walter *et al*. 1987), the rate of neural crest cell migration is not slower on a uniform ephrin substrate, but rather they act as directional repellents when presented at boundaries or in a gradient (Krull *et al*. 1997; Wang & Anderson 1997). Furthermore, *in vivo* blocking experiments in chick trunk explants show that EphB–ephrin-B interactions are required to prevent neural crest cells from entering the posterior half of somites (Krull *et al*. 1997). However, a null mutation in *ephrin-B2* does not affect neural crest or motor axon pathfinding, and this may be due to the continued presence of other guidance cues in somites (Wang *et al*. 1998).

Segmental migration of neural crest also occurs in the branchial region of vertebrate embryos, from rhombomeres to specific branchial arches where they differentiate to form specific patterns of bones and cartilage (Lumsden *et al*. 1991; Sechrist *et al*. 1993; Birgbauer *et al*. 1995; Kontges & Lumsden 1996; Saldivar *et al*. 1996). There is evidence from transplantation experiments and studies of *Hox* gene expression for both segmental specification and plasticity in the A–P identity of branchial neural crest cells (Noden 1983; Hunt *et al*. 1991, 1998; Saldivar *et al*. 1996). In an analogous manner to that discussed above (§ 7) for the hindbrain, the targeted migration of cells may act together with local signals regulating identity to maintain A–P patterning of the branchial arch neural crest.

In *Xenopus* embryos, premigratory branchial neural crest is segmented into three adjacent groups of cells that are destined to enter the first, second and third plus fourth arches, respectively (Sadaghiani & Thiebaud 1987). The complementary expression of ephrin-B2 in second-arch neural crest and mesoderm, and of EphA4 plus EphB1 in third-arch neural crest and mesoderm, has been implicated in the targeted migration of cells (Smith *et al*. 1997). After blocking or ectopic activation of these Eph receptors, there is an abnormal migration of third-arch neural crest cells into adjacent territory, consistent with ephrin-B2 acting to restrict these cells from intermingling with second-arch neural crest.

9. ROLES AT MULTIPLE STAGES OF PATTERNING

Somite formation occurs progressively along the A–P axis by the aggregation of groups of mesenchymal cells to form epithelial balls. Each somite is subdivided into anterior and posterior halves that are demarcated by a morphological boundary (Keynes & Stern 1984). As each somite differentiates, the sclerotomal component (presumptive cartilage) becomes mesenchymal, yet its segmentation is maintained to later form the repeated vertebrae. Restrictions to cell intermingling may therefore stabilize the distinct identity of somite derivatives along the body axis, and of the anterior and posterior half of each somite that contribute to distinct parts of each vertebra (Goldstein & Kalcheim 1992). Intriguingly, there is a complementary expression of ephrin-B2 in the posterior half of somites (Bergemann *et al*. 1995; Krull *et al*. 1997; Wang & Anderson 1997) and of EphA4 in the anterior half of forming somites (Nieto *et al*. 1992; Irving *et al*. 1996) in the chick and mouse, and a similar expression of these genes occurs in zebrafish embryos (Durbin *et al*. 1998). Furthermore, overexpression in zebrafish embryos of truncated or full-length ephrins that will ectopically activate EphA4 leads to the disruption of somite boundaries (Durbin *et al*. 1998). The reciprocal expression of Eph receptors and ephrins may therefore have a role, analogous to that in the hindbrain, in restricting intermingling between the anterior and posterior halves of somites.

Taken together with the studies of trunk neural crest and motor axon migration, these findings show that expression domains of Eph receptors and ephrins act at multiple steps of patterning. At early stages, repulsion mediated by these proteins may restrict intermingling between anterior and posterior half somites. In addition to allowing correct patterning of somite derivatives, this restriction stabilizes the ephrin expression domains later used as pathfinding cues by migrating cells and axons. An analogous proposal that ephrin domains may stabilize an early pattern later used as a pathfinding cue can be made for branchial arch mesoderm in *Xenopus* embryos (Smith *et al*. 1997), and for the countergradients of ephrins and Eph receptors in the tectum (Connor *et al*. 1998).

10. POTENTIAL ROLES IN CELL ADHESION

There is accumulating evidence that in neuronal growth cones, Eph receptor activation restricts growth cone movement by triggering a local depolymerization of the actin cytoskeleton leading to a collapse response. It seems likely that collapse of filapodia of neural crest cells (Jesuthasan 1996) could also be triggered by Eph receptor activation. However, it is not known whether such responses occur in epithelial tissues such as the hindbrain. There is some evidence that Eph receptors and ephrins could cause de-adhesion by regulating the function of cell adhesion molecules (Winning *et al*. 1996; Zisch *et al*. 1997; Jones *et al*. 1998). Furthermore, although Eph receptor activation can drive cell sorting in hindbrain segments (Xu *et al*. 1999), *in vitro* sorting of cells from odd and even rhombomeres requires cell adhesion molecules (Wizenmann & Lumsden 1997). One possibility is that an adhesive system that is uniformly expressed is locally regulated by activation of Eph receptors or ephrins. Alternatively, differentially expressed cell adhesion molecules may act in parallel with Eph receptors and ephrins.

In contrast to the repulsion or de-adhesion of cells observed in a number of systems, Eph receptor activation has been found to increase cell adhesion in some situations. Activation of Eph receptors with clustered soluble ephrins leads to an assembly of endothelial cells in culture into capillary-like networks (Stein *et al*. 1998), and promotes angiogenic sprouting (Adams *et al*. 1999). Intriguingly, the assembly of endothelial cells only occurred after clustering of ephrins into complexes greater than dimers, suggesting that higher-order clustering of Eph receptors may trigger a cellular response distinct from dimerization (Stein *et al*. 1998). Recent work has shown that Eph receptor activation can increase cell adhesion to extracellular matrix via integrins (Huyn Do *et al*. 1999). These findings raise the important question as to what underlies repulsion versus adhesion responses to Eph receptor activation. One explanation could be that this is due to a cell type-specific response. However, as discussed below recent studies in the retinotectal system suggest another possibility.

Although many studies have emphasized the role of complementary expression of Eph receptors and ephrins, it is now clear that overlaps in expression occur in a number of tissues (Flenniken *et al*. 1996; Connor *et al*. 1998; Sobieszczuk & Wilkinson 1999). One such site occurs in the retina, in which uniform expression of EphA4 overlaps with ephrin-A5 in axons in the anterior retina, leading to persistent receptor activation in these axons (Connor *et al*. 1998). Analysis of the effects of removing or ectopically expressing ephrin-A5 on axonal behaviour in stripe assays reveals that persistent Eph receptor activation desensitizes growth cones to exogenous ephrin, such that they navigate further up the ephrin gradient in the tectum (Hornberger *et al*. 1999). A similar conclusion can be drawn from experiments in which retinal axons encounter artificial gradients of ephrins in stripe assays (Rosentreter *et al*. 1998). Based on these findings, it will be interesting to determine whether the persistent activation of Eph receptor at other sites of overlap with ephrins desensitizes a repulsion response. An intriguing possibility is that below the threshold level for repulsion, persistent Eph receptor activation leads to an adhesive response (Huyn Do *et al*. 1999).

11. CONCLUDING PERSPECTIVES

In conclusion, studies of Eph receptors and ephrins have shown that they have important roles in morphogenesis, in which they regulate both repulsion and adhesion responses that establish or stabilize patterns of cellular organization. These advances raise many important questions. For example, do Eph receptors and ephrins act in parallel with, and/or regulate, cell adhesion molecules? What are the intracellular transduction pathways activated by Eph receptors and ephrin-B proteins, and what underlies repulsion versus adhesion responses? Do ephrin-A proteins transduce signals? Do different family members trigger the same or different responses? It is likely that important insights into their roles in morphogenesis will come from further dissection of biochemical pathways,

systematic genetic analysis in amenable systems such as *Drosophila* and *C. elegans*, as well as studies of cellular responses *in vivo*.

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