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The role of the Eph-ephrin signalling system in the regulation of developmental patterning

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ABSTRACT The Eph and ephrin system, consisting of fourteen Eph receptor tyrosine kinase proteins and nine ephrin membrane proteins in vertebrates, has been implicated in the regulation of many critical events during development. Binding of cell surface Eph and ephrin proteins results in bi-directional signals, which regulate the cytoskeletal, adhesive and motile properties of the interacting cells. Through these signals Eph and ephrin proteins are involved in early embryonic cell movements, which establish the germ layers, cell movements involved in formation of tissue boundaries and the pathfinding of axons. This review focuses on two vertebrate models, the zebrafish and mouse, in which experimental perturbation of Eph and/or ephrin expression *in vivo* have provided important insights into the role and functioning of the Eph/ephrin system.

KEY WORDS: *Eph receptor, ephrin, signalling, zebrafish, mouse knockout*

Introduction

The interaction of Eph and ephrin proteins on opposing cells triggers bi-directional signals that have been shown to regulate the cellular movements underlying critical events of developmental patterning (Wilkinson, 2001). The expression of Eph and ephrin proteins is highly regulated during development and thereafter declines to low levels in most cases. However, there is increasing evidence that these molecules can be re-expressed in some circumstances. A body of evidence supports a role in tumour formation (Dodelet and Pasquale, 2000) but the role of Eph and ephrin proteins in other pathologies remains relatively unexplored. The focus of this review is the role of these proteins in the regulation of the events of embryogenesis. Other roles in regulating post-embryonic events, in particular pathological processes, will also be reviewed where appropriate.

Eph and Ephrin Genes in Vertebrates

The structural and functional conservation of receptor tyrosine kinases (RTK) through evolution extends to both vertebrate and invertebrate phyla. Eph genes, with their distinct structural features and functional roles, appear to have arisen very early suggesting that they represent the earliest evolutionary split from RTK, occurring prior to the appearance of vertebrates. The Eph and ephrin proteins have diversified extensively during evolution of the vertebrate body plan. In *Caenorhabditis elegans* (*C. elegans)* and Drosophila there is a single Eph gene compared with fourteen receptors identified in vertebrates making this the largest subfamily of RTK (Lemke, 1997). Many RTK bind soluble ligands and regulate growth and differentiation. In contrast, the ligands for Eph receptors are cell membrane proteins (ephrins) and activation of Eph receptors by ephrin binding does not promote proliferation and differentiation (Klein, 2001). Indeed, there is evidence that Eph signalling either indirectly or directly suppresses proliferation (Conover *et al.*, 2000; Miao *et al.*, 2001). The membrane associated ephrin proteins number nine in vertebrates, falling into two distinct groups: the A group of six glycosylphosphatidyl inositol (GPI)-linked proteins and the B group of three trans-membrane proteins (Lemke, 1997; Menzel *et al.*, 2001).

Amongst vertebrates, inter-species homologues are readily identified through their very high degree of sequence conservation, particularly in key functional domains. Human EphA3, for example,

Abbreviations used in this paper: RTK, receptor tyrosine kinase; sh, soluble human.

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is 96% identical to its mouse homologue at the amino acid level, but this increases to 99% identity within the ephrin-binding domain (Lackmann *et al.,* 1998). By extending this principle to other species, it has proved relatively easy to define homologies in non-mammalian species. The failure to do so in some cases has cast doubt on the notion that the Eph family of fourteen receptors is identical in all vertebrates. In particular *EphB5*, which was isolated in chicken (Soans *et al.,* 1996), has not been found in mammals by either direct experiments or *in silico* searching of currently available genome resources. Conversely, *EphA1*, which was originally described in human (Hirai *et al.,* 1987) and mouse (Lickliter *et al.,* 1996), has not been identified in other vertebrates. We have searched extensively for sequences homologous to human and mouse EphA1 in the zebrafish genome, probing both genomic and a number of cDNA libraries with mouse EphA1 probes. The cDNAs isolated show close homology to known mammalian Eph cDNAs, but of the many (>50) clones analysed none resemble *EphA1*. These studies have been supplemented by reverse transcriptase polymerase chain reaction (RT PCR) using degenerate Eph primer strategies (Lickliter *et al.,* 1996), again yielding a number of products with close sequence homology to mammalian Eph receptor sequences but with highest homologies to Eph proteins other than EphA1. We recently interrogated the complete genome of two pufferfish, *Tetraodon nigroviridis* (http://www.genoscope.cns.fr/externe/tetraodon) and Fugu (http://fugu.hgmp.mrc.ac.uk) for EphA1-related sequences. Pufferfish diverged from zebrafish approximately 150 million years ago, reportedly after an independent genome duplication event in the teleost lineage. No *EphA1* related sequences were detected in either search but homologues of other Eph receptors were readily identifiable. Another significant observation emerged from the analysis of the genomic organization of the mouse (Coulthard *et al.,* 2001) and human (Owshalimpur and Kelley, 1999) *EphA1* loci. This demonstrated that both mammalian sequences contained an extra intron which does not occur in other Eph genes (Connor and Pasquale, 1995; Lackmann *et al.,* 1998). Based on exon phase analysis this difference appears to reflect a subsequent intron insertion (Coulthard *et al.,* 2001). Taken together with the gene searching experiments this suggests that EphA1 may not occur in all vertebrates and thus may be a late product of evolution, possibly restricted to mammals.

At least one chicken ephrin, *ephrin A6* (Menzel *et al.,* 2001), may also have no mammalian homologue. Searching of nucleotide and genome databases show that the closest mammalian genes are *ephrin A2, ephrin A3* and *ephrin A4*. As the closest match to *ephrin A6* is chicken *ephrin A2*, it is tempting to speculate that *ephrin A6* has arisen through gene duplication during the evolution of the avian lineage.

The most striking example of Eph diversity is within the bony fish (teleosts) which have undergone a further genome duplication, over and above the two occurring in the evolution of all vertebrates. This event is estimated to have occurred 150 million years ago. In zebrafish a number of ephrin genes have now been described. Through homology analysis it is relatively simple to identify homologues of genes in other vertebrate species. A partial list of Eph and ephrin related zebrafish sequences is shown in Table I, showing that for at least two Eph and two ephrin proteins there are two or more separate sequences which can be most closely aligned to a single mammalian gene. For example ephrin B2a and ephrin B2b are very similar in sequence, both to each other and to mammalian ephrin B2 (Picker *et al.,* 1999). Analysis of expression indicates that these two genes have restricted individual expression patterns when compared with mammalian ephrin B2, but the expression of both covers all analogous embryonic events believed to involve the single ephrin B2 protein during mammalian development.

Details of the evolution of Eph receptors from an ancestral gene (reflected by a single gene in *C. elegans* and Drosophila) to the full complement in different vertebrate lineages will only be clarified with the complete sequences of the many vertebrate genomes now under investigation. Similarly, there are three GPI-linked ephrins in *C. elegans* (Wang *et al.,* 1999) and probably only one in Drosophila compared with nine ephrins described in vertebrates. The possible significance of such differences in explaining the differences between scaly, furry and feathery vertebrates is intriguing but at this stage speculative.

Eph and Ephrin Activation and Signalling: Forward and Reverse

The Initial Interaction

Analysis of Eph and ephrin protein-protein interactions show that many of the receptors show cross-reactivity which is usually restricted to either GPI-linked or transmembrane ephrins (Gale *et al.,* 1996; Flanagan and Vanderhaeghen, 1998). These observations led to the classification of receptors into A or B categories depending on their preference for binding of GPI-linked or transmembrane ephrins respectively (Lemke, 1997). This early notion that a single Eph protein could bind with similar affinity to all ephrins of a certain class has proven to be inaccurate. Indeed, some interactions are relatively specific, for example EphB4 binds strongly to ephrin B2 but weakly to other ephrins (Gerety *et al.,* 1999). At the other extreme EphA4 binds members in both ephrin A and ephrin B subgroups with comparable affinities (Gale *et al.,* 1996) and, as discussed below, probably binds the ephrin B family in many of its most critical developmental roles. Even those receptors, which appear cross-reactive, exhibit an ordering in affinities of interaction with preferred high affinity ephrin interactions. An example is EphA3 which binds several A ephrins and even shows weak binding to B ephrins but binds ephrin A5 with a much higher affinity than other ephrins (Lackmann *et al.,* 1997). Individual Eph-ephrin interactions have been shown to have a strict one to one stoichiometry (Lackmann *et al.,* 1997). The binding affinity is principally determined by the rate of dissociation and thus critically determines the average half life of a particular Eph/ephrin complex. It follows that this in turn determines the probability of oligomerization of Eph-ephrin complexes, an essential requirement for triggering of autophosphorylation and signal transduction (Stein *et al.,* 1998).

Signalling – Regulation of Form and Movement

Once initiated, Eph and ephrin signal transduction mechanisms converge on the regulation of processes involved in cell shape, adhesion and movement (Xu, Mellitzer, and Wilkinson, 2000). Many Eph and ephrin signals modify the cytoskeletal architecture of the cell through recruitment and/or activation of signalling proteins directly involved in regulating cytoskeletal organization (Ellis *et al.,* 1996; Holland *et al.,* 1997; Dodelet *et al.,* 1999; Wahl *et al.,* 2000; Shamah *et al.,* 2001; Miao *et al.,* 2001; Yu *et al.,* 2001; Schmucker and Zipursky, 2001; Lai *et al.,* 2001). Eph-ephrin signals also modulate the function of integrin adhesion molecules

(Huynh-Do *et al.,* 1999; Zou *et al.,* 1999; Miao *et al.,* 2000; Becker *et al.,* 2000; Hattori, Osterfield, and Flanagan, 2000; Davy and Robbins, 2000; Huai and Drescher, 2001; Gu and Park, 2001). They are themselves regulated by cadherins (Zantek *et al.,* 1999; Orsulic and Kemler, 2000) but may also have a role in regulating cadherins (Winning, Scales, and Sargent, 1996).

Eph protein signalling occurs predominantly via phosphorylation of critical tyrosine residues in the highly conserved juxtamembrane loop (Wybenga-Groot *et al.,* 2001). The phosphorylation of this loop enables binding of second messenger proteins which initiate the signalling cascade. However, a body of evidence is emerging which shows that this is not always the case. The finding that in EphB6 the kinase domain has critical mutations which render it non-functional (Gurniak and Berg, 1996) suggested either that EphB6 uses alternate signalling pathways or has a purely adhesive role. Similar conclusions came from analysis of the binding of EphA7 splice variants to ephrin A5 during neural tube closure (Holmberg, Clarke, and Frisen J., 2000). Expression of kinase defective splice variants switched the EphA7-ephrin A5 response from repulsion to adhesion in this developmental process. Whilst it was proposed that this was the result of a dominant negative effect of the truncated splice variants, it is possible that this switch involved kinase-independent functions of the cytoplasmic domains. Such a mechanism has now been described for EphA8 in the switching of its activity from cell repulsion to cell adhesion (Gu and Park, 2001). In this situation the EphA8 activation triggers phosphorylation-independent binding of the p110γ isoform of PI-3 K in the juxtamembrane region, resulting in transduction of a phosphatidylinositol-3 kinase (PI-3K)-dependent signal, which enhances integrin adhesion to fibronectin.

Apart from Eph-mediated signals, soon after their discovery it became evident that ephrins were themselves signal transducers (Holland *et al.,* 1996). A central role for the conserved PDZ motif in the ephrin B cytoplasmic tail in protein interaction and membrane localization was established (Torres *et al.,* 1998; Bruckner *et al.,* 1999; Lin *et al.,* 1999). Ephrin B-mediated signalling through the PDZ-RGS3 protein regulates cerebellar granular cell guidance through modulation of the chemokine receptor for SDF-1 (Lu *et al.,* 2001). B ephrin signalling can also occur through phosphorylation of highly conserved cytoplasmic domain tyrosine residues that enable binding of the Grb4 SH2/SH3 (Cowan and Henkemeyer, 2001). The finding that ephrin A proteins, localized within lipid raft membrane micro-domains, also deliver signals has extended the possibility of bi-directional signalling to all Eph-ephrin interactions (Davy *et al.,* 1999; Davy and Robbins, 2000; Huai and Drescher, 2001).

In many situations Eph-ephrin signalling results in de-adhesion, collapse of cell processes and cell detachment, implying a role in contact repulsion. As the high affinity interaction between Eph and ephrin proteins forms a relatively stable linkage between opposing cells, it is necessary to disrupt this bridge before opposing cells can disengage. One mechanism for this was provided by the discovery that ephrin proteins bind a protease which is activated by Eph-ephrin signalling (Hattori, Osterfield, and Flanagan, 2000). Thus, activation of Eph-ephrin signalling results in cleavage and shedding of the ephrin exodomain allowing cell detachment to occur.

In some cases Eph-ephrin signals can be pro-adhesive, particularly through upregulation of integrin-mediated adhesion to extracellular matrix proteins (Bohme *et al.,* 1996; Jones *et al.,* 1998; Davy *et al.,* 1999; Davy and Robbins, 2000; Gu and Park, 2001). In different settings, apparently determined by receptor density, EphA8 can mediate either positive (Gu and Park, 2001) or negative (Choi and Park, 1999) effects on integrin function.

TABLE I

ZEBRAFISH HOMOLOGUES OF MAMMALIAN EPH AND EPHRIN

Mammalian	**Zebrafish homologue**
EphA2	EphA2 (rtk6*)
EphA3	EphA3 (zEphA3[+#])
EphA4	EphA4a (rtk1*), EphA4b (rtk2*), EphA4c (rtk4*) EphA4d (zephR23[#])
EphA5	EphA5 (rtk7*)
EphA7	EphA7 (zjc_ephA7[#])
EphB2	EphB2 (zephR20[#])
EphB4	EphB4a (rtk5), EphB4b (zephR5[#], rtk8)
ephrin A1	Ephrin A1 (L1*)
ephrin A2	Ephrin A2 (L3*)
ephrin A5	ephrin A5a(L4*/zephA5a[#]), ephrin A5b (L2*/zephA5b[#])
ephrin B1	ephrin B1 (zephL1[#])
ephrin B2	ephrinB2a (L5*), ephrinB2b (zephL8[#])
ephrin B3	ephrinB3 (zephL4[#])

Origin of isolates: *C. Brennan, N. Holder – University College London, UK; [#]J. Chan, T. Roberts – Dana Farber Cancer Institute, Boston; [+]M. Power, M. Down, A. Boyd - Queensland Institute of Medical Research, Australia.

Analysis of Eph and Ephrin Proteins in Zebrafish

Whilst the mouse is a well-studied developmental model it has limitations, particularly in the analysis of the early events of development. As a developmental model, zebrafish (*Brachydanio rerio*) offer a unique tool for analysis of the spatially and temporally restricted expression patterns of developmentally regulated genes. One desirable feature of zebrafish development is rapid embryogenesis, with the appearance of major organ rudiments at 24 hours post fertilization (hpf) and completion of development occurring around 72 hpf. Zebrafish embryos are externally fertilized and essentially transparent, enabling visualization of cells at all stages of development and rendering embryos amenable to techniques such as cell-fate mapping, transplantation of tissues and cells, whole-embryo immunohistochemistry and *in situ* transcript hybridization. External embryonic development also allows manipulation of embryos independently of the mother, a property exploited during chemical treatments such as ethylnitrosourea (ENU) mutagenesis. Additionally, zebrafish produce large numbers of progeny with synchronous development, while non-viable embryos are not subject to re-absorption and remain visible for observation. Zebrafish mature at 4 months and can be maintained at the high population densities required for large-scale mutation screening.

There is one apparent difficulty with the zebrafish model with respect to extrapolating results in the fish to mammalian systems. As members of the taxonomic superorder Teleosti, it appears that the zebrafish lineage experienced an additional partial tetraploidization event (Amores *et al.,* 1998). The resulting duplicate genes, where they are conserved, present a complication to the use of mutagenesis studies in zebrafish as mutations in one paralogue can be disguised by the unaffected function of other paralogues, thereby concealing mutagenic phenotypes from analysis. On the other hand, gene duplications resulting in functional divergence of paralogues such that one gene retains the classical function and the duplicate acquires new functions, and the conservation of synteny between zebrafish and mam-

mals, offers an inimitable opportunity to examine the acquisition, loss and maintenance of gene functions through evolution. In the succeeding paragraphs we will attempt to show that the potential difficulties of the increased complexity of the zebrafish genome have been outweighed by the contribution zebrafish studies have already made to the analysis of Eph and ephrin gene function.

Detailed analyses of Eph and ephrin gene expression have been performed in zebrafish, Xenopus, mouse, rat and chicken embryos. Just as structure and function is highly conserved, expression is very similar for each homologue, consistent with their role in controlling specific developmental events. Our laboratory has had a long-term interest in EphA3, having isolated human EphA3 from a pre B cell leukaemia (Wicks *et al.,* 1992). Using a human *EphA3* probe we isolated a near full length zebrafish EphA3 cDNA. The inferred amino acid sequence of the ligand binding domain is shown in Fig. 1 compared with the same region in human, mouse and chicken. Strikingly, all four sequences are identical at 219/238 residues and the zebrafish sequence shows 94% identity with the human sequence. This sequence similarity, taken together with evidence of functional equivalence in studies of the binding of EphA3 to human and zebrafish ephrin A5 (Oates *et al.,* 1999), imply a critical role for EphA3 during evolution.

Analysis of the role of the Eph/ephrin system in early embryogenesis has been carried out using dominant negative strategies. In our studies capped mRNA encoding soluble forms of ephrin A5, ephrin A2 and EphA3 were injected into 1-2 cell stage zebrafish embryos and the early events of development analysed by Nomarski microscopy and *in situ* hybridization (Oates *et al.,* 1999). As shown in Fig. 2, the injected embryos show failure of convergence and subsequent disruption of the neural tube, somites and notochord. These and other results demonstrate a role for this signalling system in gastrulation and in the subsequent convergence movements leading to formation of the prechordal plate and notochord (Oates *et al.,* 1999; Chan *et al.,* 2001). *In situ* experiments using myoD probes showed that somitogenesis was disrupted, often to the degree of complete failure. These effects might have been explained by the failure of correct convergence movements but subsequent studies show that the Eph-ephrin system also has a role in specifying somite polarity and boundary formation (Durbin *et al.,* 1998; Durbin *et al.,* 2000).

Bi-directional signalling is of great importance in explaining how Eph and ephrin proteins function in cell guidance and tissue boundary formation within the developing embryo (Klein, 1999). Perhaps the most intensively studied event in zebrafish development is the formation of the brainstem structures, in particular the hindbrain rhombomeres. The midbrain-hindbrain boundary has been shown to provide a crucial organizer function in zebrafish, without which ordered gradients of ephrin expression in the tectum are ablated (Picker *et al.,* 1999). On the hindbrain side of the boundary, a pattern of segmentally-restricted expression of Ephs and ephrins in complementary rhombomeres is apparent, which is essentially the same in zebrafish, Xenopus and mouse embryos (Xu *et al.,* 1995). Injection of ephrin-B2 mRNA into zebrafish embryos causes mosaic expression throughout the hindbrain, allowing ephrin-B2 to interact with EphA4, EphB2 and EphB3 expressed on adjacent rhombomere cells. This results in random dispersal of ephrin-B2 positive cells throughout even numbered rhombomeres, and movement of ephrin-B2 positive cells to the boundaries of rhombomeres 3 and 5, demonstrating the cell-repulsive effects of Eph-ephrin expression in rhombomere boundary formation. The role of bi-directional Eph/ephrin signalling in this process has been further elaborated by later *in vivo* (Theil *et al.,* 1998; Xu, Mellitzer, and Wilkinson, 2000) and *in vitro* (Mellitzer, Xu, and Wilkinson, 1999) studies. While these studies focused on rhombomeres 1-5 and on the role of EphA4 interacting with ephrin B ligands it is clear that in the caudal hindbrain EphB4 is involved in boundary formation, probably through interaction with ephrin B2a (Cooke *et al.,* 2001).

The role of graded EphA and ephrin A protein expression in the retina and superior colliculus in the regulation of axonal guidance and thus the establishment of the retino-collicular map was initially defined in chicken (Cheng *et al.,* 1995; Drescher *et al.,* 1995). It has been clearly shown that this process is controlled by similar proteins, functioning in an analogous fashion in both zebrafish

Fig. 1. Alignment of EphA3 amino acid sequences of the ligand binding domain. *The zebrafish, chicken, mouse and human sequences were aligned using the Vector NTI software program (Informax Inc., Bethesda, MD). Residues identical in all four sequences are shown as white on a black background. Residues identical at 3/4 positions are shown with a dark grey background and those identical at 2/4 positions with a light grey background.*

(Brennan *et al.,* 1997) and mouse (Feldheim *et al.,* 2000). Ephrin A3 expression in the posterior zebrafish tectum, however, is not imitated by corresponding expression in the mouse midbrain, implying that evolution of the vertebrate brain occurred with a change in the role of ephrin A3 in neural development (Hirate *et al.,* 2001). Despite some variations in the individual players, these and other examples illustrate a molecular mechanism common to Eph and ephrin proteins. Tightly regulated expression and resulting temporally-restricted Eph-ephrin interactions provide critical cues which define the path of migrating cells during key developmental patterning processes (Gale *et al.,* 1996).

Uncovering Eph-ephrin Function through Gene Knockout Studies

The power of the zebrafish model is limited by the lack of a gene knockout technology. This problem has been partly resolved by the introduction of morpholino antisense methodology (Corey and Abrams, 2001). The short timeframe of zebrafish development has meant that morpholino antisense oligonucleotides can successfully phenocopy known zebrafish gene loss of function mutants. Nevertheless, there are methodological limitations to this approach; gene deletion is the only certain method of analysing loss of function.

In *C. elegans* and Drosophila, transposon-based methods of introducing loss of function mutations can be used in analysis. Analysis of such mutants of Vab-1 and its ephrin ligands in *C. elegans* showed that worms expressing a kinase-deleted receptor had a less severe phenotype than full Vab-1 knockout animals. This implied a role for kinase-independent function of the Vab-1 Eph protein (George *et al.,* 1998; Wang *et al.,* 1999; Chin-Sang *et al.,* 1999). This provided the first evidence that ephrin A signalling might be triggered by interaction with Eph proteins and suggested that in some situations the Eph signal was not required. In vertebrates the method of choice for generation of loss of function mutations is targeted gene inactivation through homologous recombination in embryonic stem cells, a method that is in practical terms restricted to the mouse. The current mutants of Eph and ephrin genes are summarised in Table II. It is evident that many of the receptor mutants have no obvious phenotype or that the defects are relatively mild and restricted. In some situations this lack of phenotype might be explained by functional redundancy between Eph proteins. In the case of *EphB2* (Henkemeyer *et al.,* 1996) and *EphB3* (Orioli *et al.,* 1996) the individual mice had no obvious phenotype although axon guidance defects were evident in the anterior commissure and corpus callosum respectively. However, when these mice were crossed, a severe phenotype emerged with failure of anterior commissure and corpus callosum formation plus other axon guidance defects and gross anatomical defects in the midline cranio-facial structures which resulted in early post-natal death (Orioli *et al.,* 1996). In contrast, the ephrin mutations tend to be associated with more severe phenotypic changes. Both Eph- and ephrin-mediated signals are implicated in these processes as mice expressing kinase-deleted EphB2 and B3 receptors had a less severe phenotype than complete double-knockout mice. These kinase deletion experiments demonstrate a role for ephrin signals but not necessarily for the "reverse" Eph signal (Birgbauer *et al.,* 2000). Further examination of the EphB2-/- mutation (Cowan *et al.,* 2000) on a CD1 strain background uncovered a hyperactive circling locomotion phenotype due to abnormal innervation of the inner ear epithelium and a defect in endolymph production in the semicircular canals.

The *EphA4* mutant, the only one with an immediately recognizable phenotype, demonstrates the role of the Eph/ephrin system in the development of tracts that cross the midline axis. The *EphA4*-/- mouse has been shown to have two distinct anatomical defects: a failure of axon guidance of corticospinal neurons resulting in a hopping, kangaroo-like gait and a defect in formation of the anterior commissure (Dottori *et al.,* 1998). Further analysis of these two developmental defects was carried out by generating *EphA4* mutant knock-in mice. Two lines of mice with intact *EphA4*

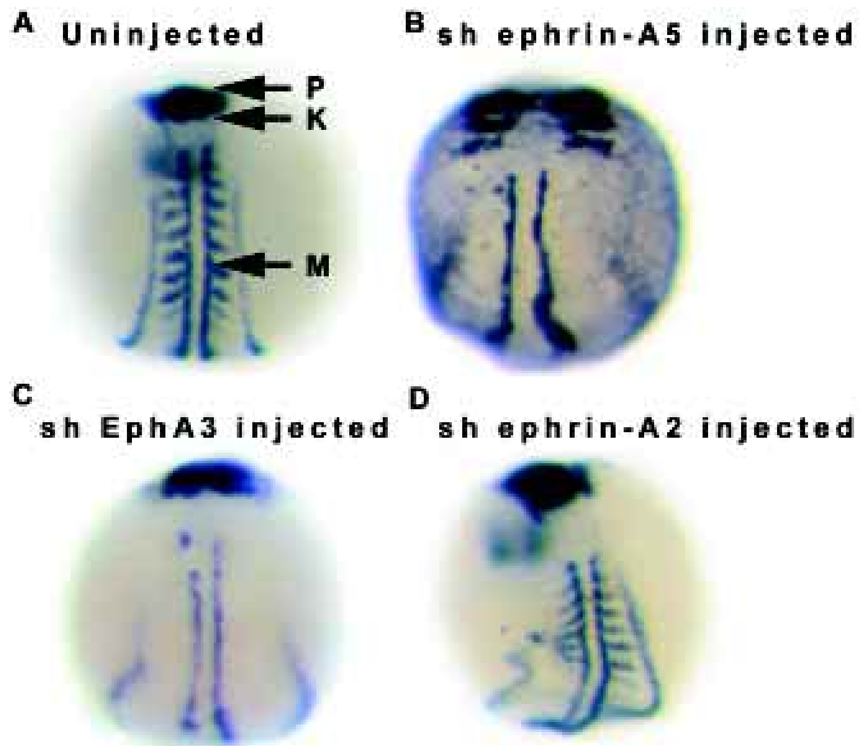

Fig. 2. *In situ* hybridisation of soluble human (sh) EphA3, sh ephrin-A2 and sh ephrin-A5 injected zebrafish embryos. *The range of dramatic convergence defects resulting from the dominant negative effects of soluble Eph and ephrin injections into zebrafish embryos are phenotypically similar for sh EphA3, sh ephrin-A2 and sh ephrin-A5. Following injection, embryos were allowed to develop for 12 h, then fixed for simultaneous* in situ *hybridisation with* myoD *(M),* krox20 *(K) and* pax2.1 *(P) DIG-labelled riboprobes.* **(A)** *Uninjected embryo at 12 hpf showing normal expression of* pax2.1 *in the MHB,* krox20 *in the 3rd and 5th hindbrain rhombomeres and* myoD *in the adaxial and paraxial mesoderm.* **(B)** *sh ephrin-A5 injected embryo at 12 hpf showing incomplete convergence of the entire body axis including the brain and notochord.* **(C)** *sh EphA3 injected embryo at 12 hpf, demonstrating defective somitogenesis with absence of paraxial* myoD *expression and incomplete adaxial marker expression.* **(D)** *sh ephrin-A2 injected embryo at 12 hpf, with disturbance of paraxial mesoderm and kinking of the notochord.*

TABLE II

MOUSE MUTATIONS OF EPH AND EPHRIN GENES

	Method	**Phenotype**	**Anatomical defect**	**Comments**
EphB2 *et al.,* 1996)” (Henkemeyer *et al.,* 1996)	Replacement vector (*pgk-neo*) & extracellular domain-β-galactosidase fusion reporter	No discernible phenotype	Failure of formation of pars posterior of the anterior commissure	See text
EphB3 *et al.,* 1996)” (Orioli *et al.,* 1996)	Replacement vector (*pgk-neo*) kinase domain	No discernible phenotype; double mutants cleft palate with perinatal lethality anterior commissure	Absent corpus callosum (low penetrance); normal	See text
EphB2/EphB3 (Birgbauer *et al.,* 2001)	EphB2/EphB3 double KO (Orioli *et al.,* 1996)	As above	Abnormal pathfinding of retinal ganglion cells (RGC) within the retina	EphB2/EphB3 guide RGC's; EphB2 kinase domain not required
EphB2 (Cowan *et al.,* 2000)	Henkemeyer mice backcrossed on to CD1 strain	Hyperactive & circling behaviour	Inner ear epithelium fibres do not cross into contralateral ear	Abnormal amount endolymph in semicircular canals
EphA2 (Chen *et al.,* 1996)	Gene trap strategy – U3β-geo retrovirus	No obvious phenotype	No anatomical defect detected	
EphA2 (Naruse-Nakajima, Asano, and Iwakura, 2001)	Gene trap vector – ROSAN β-geo	Kinky tail	Kinky tail & ectopic vertebrae due to splitting of the notochord	EphA2 expressing notochord cells excluded from tail tip by ephrinA1
EphA4 (Dottori *et al.,* 1998)	Gene replacement *pgk-neo* of ligand binding domain	Kangaroo-like (ROO) hopping gait;	Abnormal corticospinal tracts; absent anterior commissure	See text
EphA4 (Helmbacher *et al.,* 2000)	Replacement vector Exon I; lac-Z reporter fusion	Hindlimb phenotype (“club foot”) high penetrance	Loss of dorsal hindlimb innervation (peroneal nerve); absent anterior commissure	EphA4 involved in direction of motor axons into the dorsal part of the hindlimb
EphA4 (Kullander *et al.,* 2001b)	EphA4 Knock-in strategy – control and signalling mutants	Hopping gait	Signalling mutants: abnormal hopping gait; anterior commissure OK	See text
EphA4 (Coonan *et al.,* 2001)	Dottori mice backcrossed onto a C57BL/6 background	As described	As described	ephrinB3 expressed in midline spinal cord prevents EphA4 expressing CST axons from re-crossing the midline
ephrinB3 (Yokoyama *et al.,* 2001; Kullander *et al.,* 2001a)	Replacement vector (ephrinB3-neo); extracellular domain-lacZ fusion receptor; extracellular domain truncated	ephrinB3-neo hopping gait (similar EphA4-KO); ephrinB3-lacZ normal gait	Defective CST pathfinding	ephrinB3 expressed middle of spinal cord prevents EphA4 expressing CST axons re-crossing midline; ephrin B3 forward signalling only required for CST formation
ephrinB2 (Wang, Chen, and Anderson, 1998)	Replacement vector; extracellular domain replaced lac-Z	Embryonic lethal E11 (100% penetrance)	Extensive disruption of angiogenesis in yolk sac; absence branches carotid arteries; defective trabeculation of heart; failure vascularization of neural tube	ephrin B2 expression arteries not veins required for remodelling of capillary networks
EphB4 (Gerety *et al.,* 1999)	Replacement vector; extracellular domain replaced lac-Z	Embryonic lethal E9.5 (high penetrance); similar to ephrinB2 above	Abnormal cardiac looping;	EphB4/ephrinB2 partners in angiogenesis and cardiac development
EphB4/ephrinB2 (Adams *et al.,* 1999)	ephrinB2-lacZ (Wang 1998); double mutant EphB2/EphB3 (Orioli 1996)	ephrinB2 as above; EphB2/EphB3 double KO vascular defects 30% penetrance	EphB2/EphB3 double KO similar ephrinB2	EphB2/EphB3/ ephrinB2 involved remodelling embryonic vasculature and boundary formation between arteries and veins
EphA8 (Park, Frisen, and Barbacid, 1997)	Targeted disruption Exon I –lacZ fusion receptor	No discernible phenotype	Defective superior colliculus commissural projection to contralateral inferior colliculus; Abnormal projection axons from ipsilateral superior colliculus into spinal cord	EphA8 involved axonal pathfinding from superior colliculus to contralateral inferior colliculus
ephrinA5 (Frisen *et al.,* 1998)	Replacement vector (*pgk-neo*)	Subpopulation (17%) midline defect dorsal head	Abnormal topographic mapping temportal retinal axons on tectum	Anterograde/ retrograde dye injection studies
Ephrin A2/ephrin A5 (Feng *et al.,* 2000)	EphrinA2/ephrinA5 double KO (Frisen 1998)	None described	Abnormal mapping of the phrenic nerve on diaphragm muscle	Developing skeletal muscle expresses all ephrinA receptors; involved topographic mapping of motor axons
ephrinA2/ephrinA5 (Feldheim *et al.,* 2000)	ephrinA2/ephrinA5 double KO	Not described	Severe disruption of retino-ectal topographic map.	EphA3/EphA5 probable ligands
Ephrin A5 (Prakash *et al.,* 2000)	ephrinA5 (Frisen 1998 above)	Not described	See text.	Quantitative analysis of whisker functional representation; retrograde axon tracing
ephrinA2/ephrinA5 (Lyckman *et al.,* 2001)	ephrinA2/ephrinA5 double KO	Not described	Abnormal retino-thalamic projections.	See text.

exodomains but with mutations in the kinase domain which ablated kinase function have clearly shown that the two defects are mediated by Eph and ephrin signals respectively (Kullander *et al.,* 2001b). The pioneering corticospinal tract neurones express EphA4 and the formation of this tract was shown to be dependent on intact kinase function. Expression data implied that ephrin B3 expressed at the midline of the spinal cord was the source of axon repulsion in this pathway. This signal appeared to prevent pioneering axon growth cones from re-crossing in the spinal cord once they had crossed the midline in the medulla (Coonan *et al.,* 2001; Kullander *et al.,* 2001b). Analysis of an ephrin B3 knockout mouse provides direct evidence that this is indeed the case (Kullander *et al.,* 2001a), this mouse having the same kangaroo-like gait defect but interestingly lacking the anterior commissural defect. In contrast, analysis of EphA4 knock-in mutant mice in which either the kinase was inactive or the critical juxtamembrane tyrosines were mutated showed that the formation of the anterior commissure did not require EphA4 signalling. In this case the expression data implied that formation of this structure depended on ephrin B2 and possibly ephrin B3 signalling (Kullander *et al.,* 2001b). These experiments imply that in some situations Eph or ephrin signals are only required in one direction, as depicted in Fig. 3 for the corticospinal tract and anterior commissure. It appears that in some situations Eph or ephrin proteins are required to act only in a passive anchorage role.

As a generalization, the ephrin knockouts show more extensive defects than the Eph knockouts, perhaps due to their smaller number reducing functional redundancy. The most dramatic case is that of ephrin B2, where a knockout results in embryonic lethality due a failure to pattern arteries and veins within primitive vascular plexuses (Wang, Chen, and Anderson, 1998; Adams *et al.,* 2001). In keeping with its narrow specificity for ephrin B2, the null mutation of EphB4 phenocopies the ephrin B2-/- defect (Gerety *et al.,* 1999). How ephrin B2 and EphB4 signals result in specification of arteries and veins within the primitive vascular plexus is not yet known. However, it seems likely that mutual cell repulsion may be involved in the segregation of ephrin B2-expressing from EphB4-expressing cells. These cells partition into regions fated to form arteries and veins respectively. Unlike EphB4, ephrin B2 interacts with EphA4 (see above) and several other EphB proteins, thereby participating in the regulation of other developmental events (Yue *et al.,* 1999; Munthe *et al.,* 2000; Elowe *et al.,* 2001).

The severe phenotype of ephrin B2 compares with ephrin B3 which has a significant although not so severe defect (Kullander *et al.,* 2001a). Perhaps also consistent with greater number implying more redundancy of function, the ephrin A2 and A5 knockout mice show relatively mild phenotypes (Frisen *et al.,* 1998; Feldheim (Frisen *et al.,* 1998; Feldheim *et al.,* 2000). Both ephrin A2 and A5 are expressed in the optic tectum as overlapping anterior to posterior gradients of increasing expression (Goodhill and Richards, 1999). The notion that these gradients control the mapping of retinotectal axons was supported by the analysis of ephrin A5-null mutant mice which showed mapping defects in the optic tectum and in some cases overshooting of these axons into adjacent brain structures (Frisen *et al.,* 1998). While most mice showed no obvious phenotype, in a small proportion midline neural tube closure malformations were noted. These mice show defects in cortical organization in both sensory (Prakash *et al.,* 2000) and motor areas (Yabuta, Butler, and Callaway, 2000). Ephrin A2-/- mice were also relatively normal but again mapping defects could be detected within the tectum. In contrast, the ephrin A2/A5 double knockout mouse showed severe mapping defects with an almost complete loss of the map in the anterio-posterior axis and a less severe defect in the dorso-ventral axis (Feldheim *et al.,* 2000). These mice show other abnormalities including defects in thalamic wiring (Lyckman *et al.,* 2001) and defects in muscle innervation (Feng *et al.,* 2000). EphA8-/- mice (Park *et al.,* 1996) do not display a behavioral phenotype but have a pathfinding defect in a specific population of superior colliculus neurons. These neurons innervate the contralateral inferior colliculus, which results in aberrant axonal projections into the upper spinal cord.

Future Directions

Currently there is an explosion in studies analysing signalling by Eph and ephrin proteins. Research into the role of these proteins in cancer and other diseases is another clear direction for future research. In terms of development, the investigation of the Eph-ephrin system has mainly focused on central nervous system development. It is perhaps hardly surprising that the development of such a complex organ involves a large number of molecular cues and, in particular, that virtually all the capacity of the Eph-ephrin system is employed in aspects of central nervous system patterning. While much has been achieved this area remains very active and many challenges remain.

Our own focus is on the role of Eph and ephrin proteins in the development of other organ systems. It is clear that many Eph and ephrin proteins are expressed during the development of other organs. The expression of EphB4 and ephrin B2 in vascular development was discussed above and these proteins are also implicated in haemopoietic (Inada *et al.,* 1997) and mammary gland development (Nikolova *et al.,* 1998). Indeed EphB4 is expressed widely in epithelial tissues (Bennett *et al.,* 1994).

Our own studies have focused on EphA1, which is also widely expressed in epithelial tissues (Lickliter *et al.,* 1996) and in epithelial tumours (Maru *et al.,* 1988). The similarity of EphA1 to EphA2 in both

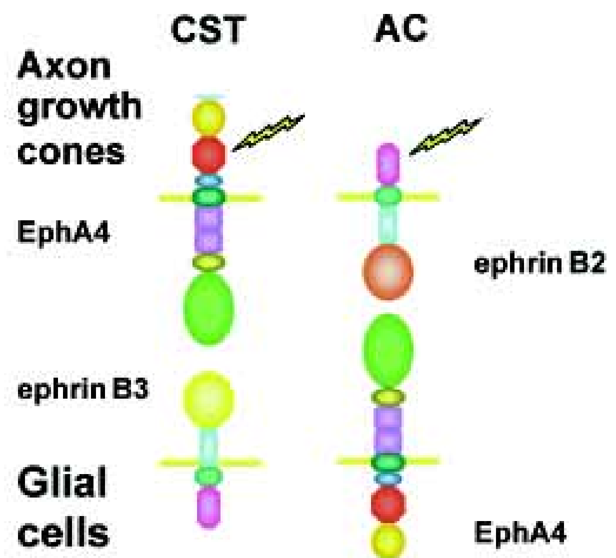

Fig. 3. Differing roles of EphA4 in the formation of the cortico-spinal tract and anterior commissure. *Lightning symbol indicates the site of active signalling. CST, corticospinal tract; AC, anterior commissure.*

epithelial tissue expression and ephrin binding affinities is highlighted by our studies which demonstrate that, whilst EphA1 binds a number of ephrin A ligands, like EphA2 it shows highest affinity for ephrin A1 (Coulthard *et al.,* 2001). These observations, taken together with the relatively mild phenotype of the EphA2-/- mouse (Naruse-Nakajima, Asano, and Iwakura, 2001), suggest that EphA1 and EphA2 may have overlapping roles in epithelial tissues. We are currently developing EphA1 null mutant mice using homologous recombination in ES cells. As in our work on EphA4 we are targeting the exons of EphA1 which encode the ligand binding domain, a strategy which led to complete knockout of expression in the case of EphA4 (Dottori *et al.,* 1998). These mutant mice should provide a powerful tool for defining the role of Eph receptors and their ligands in the development and maintenance of epithelial structures.

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