Structural Basis of Adhesion during Gastrulation and Brain Morphogenesis

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Morphogenesis, shape creation, is one of the central questions in developmental biology. It transforms a cluster of nearly identical cells in a blastula into a complex entity with structured tissues and organs. This transformation starts with gastrulation and reaches its highest complexity during brain morphogenesis. Molecular mechanisms driving morphogenesis have received great attention and cell adhesion molecules (CAMs) have been brought to the center of the stage. Here we review the structural basis of the functions of CAMs, highlighting their roles in gastrulation and brain morphogenesis. We also speculate the involvement of additional molecules such as adhesion G protein-coupled receptors (adhesion GPCRs) as novel CAMs in morphogenesis.

Blastula
An animal embryo, spherical in shape, composed of small cells derived from divisions of the fertilized ovum.

Gastrula
An animal embryo at the stage following the blastula. It is composed of three germ layers, the outer ectoderm, the middle mesoderm and the inner endoderm.

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Gastrulation is a masterpiece symphony performed by specified organ progenitors undergoing coherent morphogenetic movements. The movements of epiboly, internalization, convergence and extension transform the radially symmetric blastula into the gastrula with clear dorsal-ventral (D-V) and anterior-posterior (A-P) axes. Epiboly spreads the tissue vegetal-wards; internalization separates mesodermal and endodermal precursor cells from surface ectodermal layer; convergence drives tissue narrowing towards dorsal, and extension elongates the embryo anteroposteriorly[1]. After gastrulation, morphogenesis takes place within germ layers, tissues and organs and its complexity is championed by brain morphogenesis. Neural tissue starts out as a sheet of epithelium, which soon folds into neural tube. Within this structure, newly born neurons undergo migration to form cortical layers and cluster into functional groups. Most incredibly, synapses need to form precisely between two neurons among ten billions of neurons in the brain. Then, what's the mechanism underlying the powerful morphogenesis?

One major aspect of the answer goes to the cell surface. In 1955, Townes and Holtfreter prepared single-cell suspensions from each of the three germ layers of amphibian embryos soon after the neural tube had formed. By using embryos from species having cells of different sizes and colors, they were able to follow the behavior of cells from each layer, after cell suspensions were combined. Surprisingly, they found cells become spatially segregated after reaggregation and their final positions reflect their embryonic positions, with the ectoderm peripheral, the endoderm internal and the mesoderm in between[2]. This phenomenon can be nicely explained by "differential adhesion hypothesis" (DAH) proposed by Malcolm Steinberg. DAH reasons that the differences of the adhesive strength between cell types are what needed for sorting to occur, and the differential adhesive strength is endorsed by the differential expression of CAMs on the cell surface[3] (**Figure 1**).

There are five principal classes of CAMs: cadherin, immunoglobulin-like cell adhesion molecule (IgCAM), selectin, mucin, and integrin. Other molecules are also identified to possess adhesive properties, while mediating signal transduction. Ephrin and Eph are good examples of such molecules. Recently, a newly classified GPCR family, adhesion GPCR, has emerged as molecules with potential dual roles in cellular adhesion and signaling. Their functions in morphogenetic events are highly speculated.

STRUCTURAL BASIS OF ADHESION

Cadherins. Cadherins are characterized by the presence of cadherin repeats in their extracellular domain. Each cadherin has several tandem cadherin repeats and each of the 110-amino-acid repeats forms Greek-key β-sheet. The specific binding of three calcium ions between successive repeats rigidifies the extracellular domain to adopt an elongated crescent shape[4]. Cadherins are grouped into 5 subtypes, namely, classic cadherins, desmosomal cadherins, atypical cadherins, proto-cadherins and cadherin domain-containing proteins. The cytoplasmic domain of classic cadherins interacts with catenin complex, which anchors cadherins onto actin cytoskeleton.

In general, cadherins mediate intercellular adhesion via homophilic binding. Currently, domain-

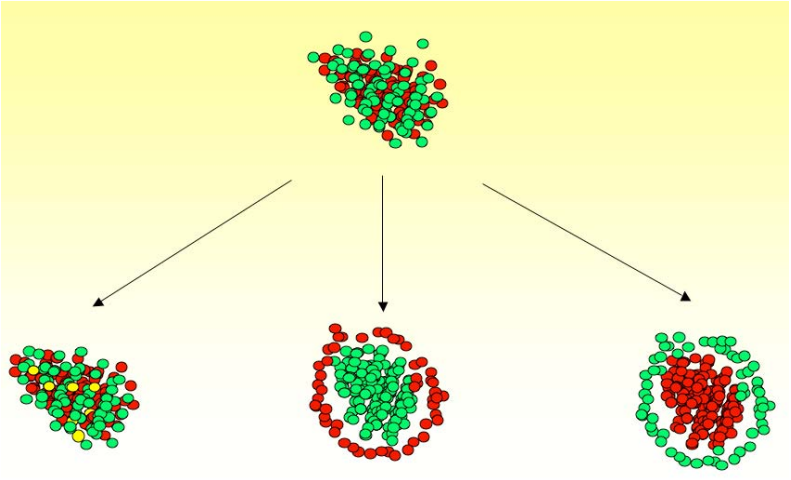

Figure 1 | **Demonstration of the DAH.** Green cells and red cells share the same cellular properties except the expression of CAMs. These two cell populations will stay in a mixture if the CAMs they express can bind to each other and endow cells with equal adhesive strength. Otherwise, the two cell populations will segregate. The more adhesive population will stay inside the less adhesive one.

swapping model is best supported by structural, biochemical studies on classic cadherins. In this model, two appose cadherins dimerize via their EC1 repeats (the distal most cadherin repeat). At the interface, the conserved Trp2 side chain from each molecule insert into the hydrophobic core of the other[5]. In support of this model, two CE1 monomer conformations were found in crystals: one with Trp2 side chain disordered[6] and the other, inactive, with the side chain inserted into its own hydrophobic pocket[7]. Furthermore, cis-dimer formation and clustering of E-cadherin have been shown to enhance its adhesive activity[8]. Not much is known about the binding of other cadherins, but differences from classic cadherins have been noted[9].

Cadherin-mediated cell sorting during morphogenesis has been an important question in the field. Early cadherin in-vitro transfection experiments suggested that the homophilic binding specificity determines sorting. However, later experiments argued that the quantity of surface-expressed cadherins determines the overall adhesive strength and is also important for cell sorting[10]. Furthermore, the observation of different conformational states of cadherin raised the possibility that cell signaling can regulate their adhesive activity.

IgCAMs. IgCAMs are CAMs with N-terminal immunoglobulin-like (Ig-like) domains. Like cadherin repeat, this Ig-like domain folds into a Greek key β-sheet. Depending on the number of β-strands, Ig-like domain can be subdivided into V-type (with 9 β-strands) and C-type (with 7 strands). The number of Ig-like domain contained in IgCAMs varies from 1 to 48. Likewise, members of this protein family have diverse mechanism of functions. Some have homophilic binding specificity, while others interact with other IgCAMs or other CAMs, such as integrin[11].

Via crystal structure studies, one common binding mechanism was found in several IgCAMs with homophilic binding specificity. IgCAM Hemolin has 4 Ig-like domains. In the crystal, these Ig-like domains bend into a horseshoe shape, with Ig-like domain 1 interacting with domain 4, and domain 2 with domain 3. Therefore it was speculated that, when two Hemolin proteins come close from opposing membranes, the Ig-like domain 1 and 2 of one Hemolin could bind to Ig-like domain 4 and 3 of the other Hemolin and vise versa[12].

One of the special traits of IgCAMs, in regards to differential adhesion, is their impressive repertoire of splicing variants. One extreme example is *Down syndrome CAM (DSCAM). DSCAM* can potentially be spliced into 38016 isoforms in *Drosophila.* Recently, the crystal structures of the Ig-like Domains of two *DSCAM* isoforms were determined. Interestingly, the different peptides generated by alternative splicing in domain 2 and 3 were pivotal to determine the homophilic binding specificity. Swapping these peptides could completely switch the binding specificity between these two isoforms[13].

Integrins. Integrins are heterodimers of two single-transmembrane subunits (α and β). There are 18 α subunits and 8 β subunits encoded in vertebrate genomes, forming at least 24 different integrins. Integrin molecules can be dissected into 3 parts: the cytoplasmic region, the membrane-proximal tailpiece and the membrane-distal headpiece. Ligand-binding specificity of integrins is encoded in the I domain (of some α subunits) or the I-like domain (of β subunits) in the headpiece[14].

Integrins can exist in different ligand-binding affinity states, corresponding to different confirmations. In the low-affinity state, the tails and cytoplasmic regions of α and β subunit associate with each other to restrain the headpiece in a bent conformation. When integrins shift into the high-affinity state, the headpiece dissociate from tailpiece to adopt an extended confirmation[15]. This shift can be induced by the presence of extracellular ligand and inside-out signaling.

The diverse roles of integrins during morphogenesis, in part, come from their ability to mediate cell-cell and cell-matrix adhesion. Integrins are able to form heterophilic interactions with multiple CAMs and cell matrix proteins, such as IgCAM, E-cadherin, fibrinogen, collagen and laminin. *Mucins and selectins.* Interactions of selectins and mucins mediate tethering and rolling adhesion of leukocytes and platelets on vascular surfaces.

Selectins are transmembrane proteins with a membrane-distal lectin domain, which binds to sLe^x on the mucin side chain in a Ca^{2+} dependent manner[16]. On the other hand, mucins are large, heavily glycosylated proteins. Their serine- and threonine-rich mucin motif is subject to extensive O-glycosylation, which decorates the main peptide chain like a bottlebrush[17]. The cytoplasmic regions of both mucins and selectins are anchored to actin skeleton.

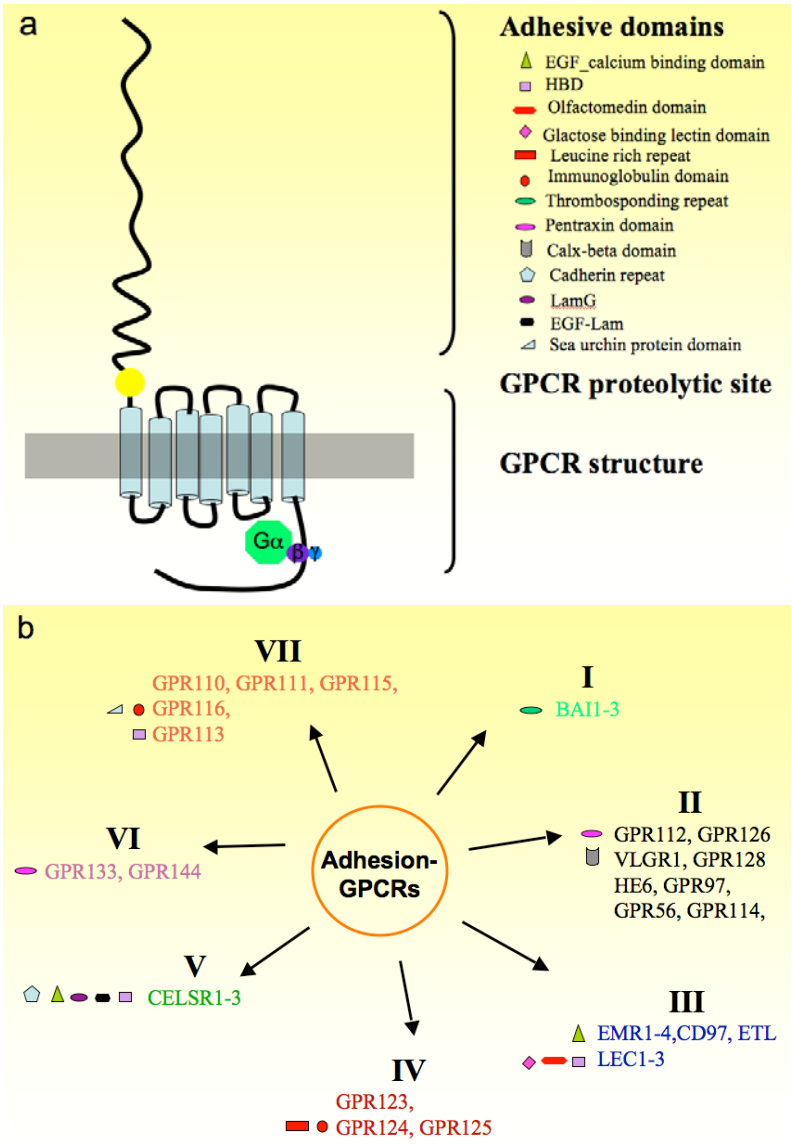

Figure 2 | **Schematic protein structure and subfamilies of adhesion GPCRs. a** | Adhesion GPCRs are natural chimeras of adhesion molecules and GPCRs. After the cleavage within the GPCR proteolytic site, the extracellular fragment and the GPCR fragment form heterodimers via non-covalent bonds. **b** | Human adhesion GPCRs are divided into seven subfamilies based on the phylogenetic study of their seven-transmembrane domain. Members of one subfamily tend to have same adhesive domains. BAI, the brain-specific angiogenesis-inhibitory receptor; VLGR, the very large G-protein-coupled receptor; HE6, Human Epididymis-specific protein 6; EMR, the EGF-like module containing receptor; ETL, the EGF-TM7-latrophilin-related receptor; LEC, lectomedin receptor; CELSR, the EGF LAG seven-pass G-type receptor.

Deletion mutants, lacking the binding sites for cytoskeleton proteins affect or eliminate rolling adhesions[18]. Interestingly, mucin-like motif is frequently seen in adhesion GPCRs. It adds potential adhesive value to adhesion GPCRs.

Ephrins and Ephs. Ephs are receptor tyrosine kinases with distinctive extracellular features. Their extracellular region, comprised of an N-terminal ephrin binding domain, an EGF-like domain and two fibronectin III motifs, is reminiscent to other CAMs. Their ligands, ephrins, are grouped into two classes: EphrinAs anchor on the plasma membrane through a glycosylphosphatidylinositol group, while EphrinBs have a transmembrane and cytoplasmic domain. Accordingly, EphrinA-binding Ephs are called EphAs, and EphrinB-binding Ephs are EphBs.

According to the crystal structure of EphB2 and EphrinB2 complex, each Eph bind to an ephrin through an expansive dimerization interface dominated by the insertion of an extended ephrin loop into a channel at the surface of the receptor. Then two Eph-Ephrin dimmers join to form a ring-like tetramer[19]. This high-affinity binding can be switched off via two mechanisms. It was discovered that interaction of EphA3 with EphrinA2 or A5 leads to cleavage of the ligand by ADAM-10 metalloproteinase, resulting in the dissociation of ligand from receptor[20, 21]. In addition, EphB-EphrinB interaction can be terminated by endocytosis of the complex into EphB- or EphrinB- expressing cells[22, 23].

The manifest effect of ephrins and Eph receptors during embryonic morphogenesis is to mediate cell segregation at the interface of their complementary expression domains or within regions of co-expression or overlapping gradients[24]. This effect provides striking example for DAH that cells with different adhesion properties would adjust their positions to maximize their bindings with cells of similar affinity.

Adhesion GPCRs. Before Adhesion GPCRs were given this name, some of them were known as LN-TM_7 or EGF- TM_7 receptors, implying that they are seven transmembrane proteins with EGF-like domains in the long extracellular N-termini. Since they are most related to secretin-receptor family (B1) in sequence, these receptors were classified as B2 family GPCRs[25]. However, the overall sequence similarity between these LN-TM_7 receptors and B1 receptors is fairly low and they differ in many aspects. In 2002, Fredriksson *et al*. proposed a new GPCR classification system, GRAFS, based on the phylogenetic analysis of the entire repertoire of the seven transmembrane regions of GPCRs. In GRAFS, LN-TM_7 receptors were for the first time grouped into a distinct family and named as adhesion GPCR[26].

GRAFS
Glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin: The five main GPCR families.

Morpholino oligonucleotides
Modified antisense oligonucleotides, which bind to the complementary RNA sequences to block RNA splicing or translation.

Later, the same group created Hidden Markov Models derived from GRAFS groups to survey the genomes from 13 species[27]. They found adhesion GPCRs exist in all animal species surveyed and there are 33 human adhesion GPCRs and about 22 zebrafish adhesion GPCRs, which is in the same range with our findings (**Figure 2**).

The long N-termini of Adhesion GPCRs are usually composed of several functional domains[28]. GPCR proteolytic site (GPS) is conserved in all Adhesion GPCRs, except *GPR123*. It is located adjacent to the first transmembrane region and contains 4 conserved cystines, one glycine and two tryptophans. The cleavage within GPS has been reported for CD97, ETL, EMR2, EMR4 and LEC1 and it was shown that the cleavage is essential for surface expression of LEC1[29]. After cleavage, the two parts form a heterodimer via non-covalent interaction. Other than GPS, adhesion GPCRs have functional domains with adhesive properties, such as cadherin repeat, EGF-like domain, Ig-like domain, leucine-rich domain and mucin-like motif (**Figure 2**). Very little is known about the interaction of adhesion GPCRs with their ligands. Nevertheless, CD97 was reported to bind the SCR repeat pf CD55 via its first two EGF-like domains[30], while bind chondroitin sulphate via its forth EGF-like domain[31]. Since more than half of adhesion GPCRs have multiple adhesive domains and all of them are highly glycosylated, they are likely to interact with more than one ligand.

So far, only GPR56 has been shown to functionally couple to $G\alpha_{12/13}$[32] and form a complex with $G_{q/11}$[33]. But G protein–coupling to other adhesion GPCRs remains a possibility. Other intracellular interacting proteins were discovered for some adhesion GPCRs. The combination of unique features supports the notion that adhesion GPCRs could act as adhesion molecules with signaling capability.

ADHESION AND GASTRULATION

From studies on zebrafish, we learned that gastrulation movements are driven by a variety of cell behaviors. Slow- and fast- directed migration and mediolateral intercalation drive convergence and extension; radial intercalation plays an important role in epiboly of deep cells, and cell movements are coupled with changes of cell shape[1]. The contributions of CAMs to these behaviors are indispensable.

E-cadherin plays widespread roles during zebrafish gastrulation. Mutations in *half baked (E-cadherin)* cause epiboly arrest, disrupted convergence & extension and failure of prechordal plate cells to elongate and migrate efficiently towards animal pole after internalization. Kane *et al*. reported that there is a radial gradient of E-cadherin expression from the deepest layer of the blastoderm (lowest expression) to the superficial layer of the blastoderm (highest expression) at shield stage. They reasoned upregulation of E-cadherin was required to maintain cells in the exterior layer after radial intercalation, since in *half baked* mutant, radially intercalated cells tend to neither change cell shape nor become restricted and often de-intercalate and move back to the interior layer[34]. By contrast, Montero *et al*. argued that embryos, injected with E-cadherin morpholino oligonucleotides to block E-cadherin expression, had reduced radial intercalation at 65% epiboly[35]. Although these two reports seemingly failed to reach a consistant conclusion, they in fact suggest that perfect strength of E-cadherin mediated adhesion is required for normal gastrulation and its slight changes might lead to different types of cell-behavioral defects.

The crosstalk between CAMs during gastrulation is another outstanding question in the field. It was first shown that protocadherin could regulate *Xenopus* gastrulation via homophilic interactions. However, Chen and Gumbiner later found more compelling evidence that paraxial protocadherin (PAPC) mediates cell sorting and influences gastrulation movements by down-regulating C-cadherin activity in *Xenopus* embryos. Among other lines of evidence, they found a dominant–negative form of C-cadherin can rescue the blastopore closure defect, caused by loss of endogenous PAPC[9]. Interestingly, crosstalk between CAMs from different families was also reported. Marsden and Desimone discovered that applying fibronectin blocking antibody or expressing a dominant- negative form of β1 integrin alters C-cadherin-mediated cell adhesion and inhibits medial-lateral cell intercalation and axial extension in gastrulating *Xenopus* embryos and explants[36]. The same group also reported that fibronectin and integrin interaction suppresses random protrusions in favor of polarized protrusions to facilitate mediolateral intercalation[37]. However, whether C-cadherin is involved in this process was not mentioned. Although the detailed mechanisms of crosstalks are still elusive, it is confirmed that C-cadherin expression level is not altered in either case. As we dig deeper, more adhesion molecules and more adhesive crosstalks ought to be discovered in the future.

ADHESION AND BRAIN MORPHOGENESIS

CAMs play diverse roles in nearly all aspects of brain morphogenesis, from neurulation to synaptogenesis. Their roles in brain morphogenesis are implied by their distinctive temporal and spatial expression pattern in the brain and justified by the phenotypes of knockout, knockdown, mutant animal models or human diseases. Among cadherins, the function of N-cadherin in the developing nervous system has been extensively studied. In zebrafish, it is

required to maintain the integrity of neuroepithelium[38] and it also plays a role in axon migration[39]. Differential combinatorial expression of type II cadherins could regulate motor neuron pool sorting[40]. And differential and combinatorial expression of protocadherins is also speculated to play a role in establishing specific neuronal connections, based on the existence of multiple splicing variants and their synaptic localization. An exciting progress has recently been made on *DSCAM*, of which the outrageous alterative splicing was mentioned above. Using mosaic analysis to mark single neurons, homophilic DSCAM-DSCAM interactions are demonstrated to be required for dendrite self-avoidance in *Drosophila* larval[41]. Another long-term favored subject in this field is the function of ephrins and Ephs during rhombomere formation. Several ephrins and their corresponding Ephs are found expressed in alternating presumptive rhombomeres. Lines of evidence demonstrate that ephrin- and Eph-mediated repulsion at rhombomere interface drives cell sorting and boundary formation[42]. However, it is not the only way Eph signaling regulates rhombomere formation. When Cooke *et al*. transplanted EphA4 morpholino-injected (MO) cells into wild-type (WT) embryos, they found that those cells could integrate with host cells in even-numbered rhombomeres (which don't express EphA4), while these transplanted cells were pushed robustly to the edges of r3 and r5 (both of which express EphA4). Reversely, when WT cells were transplanted into EphA4MO embryos, they formed pure clusters within r3 and r5. In both cases, transplanted cells maintained r3 or r5 identities within r3 and r5[43]. This experiment suggests that Eph- mediated cell adhesion within rhombomeres also contributes to cell sorting during rhombomere formation.

Rhombomere
A transiently divided segment of the developing neural tube in the area of future hindbrain.

A new aspect of the field is opened by the emergence of adhesion GPCRs. Although the majority of adhesion GPCRs are still poorly studied orphans, the critical functions of *Celsr* proteins and *GPR56* during brain development have been unveiled. *Celsr* genes are mammalian homologues of *Drosophila flamingo*. The homozygous *Celsr1* mutant embryos fail to initiate neural tube closure and have severe defect in the planar cell polarity of hair cells in the organ of corti[44]. Celsr2 and Celsr3 regulate neurite growth in an opposing manner. Celsr2 enhanced neurite growth, whereas Celsr3 suppressed it[45]. In addition, Celsr3 mediates axonal tract formation in mammals[46]. It was also uncovered that Celsrs regulate facial motor neuron migration in zebrafish[47]. The mutations in *GPR56* were first identified from patients with bilateral frontoparietal polymicrogyria[48]. Consistently, loss of *Gpr56* function in mouse results in a cobblestone-like cortical malformation[49]. Li *et al*. provided compelling evidence that GPR56 interacts with a yet unidentified ligand in the marginal zone or overlying extracellular matrix to regulate the integrity of pial basement membrane and therefore influence cortical lamination. Comparable expression profile of each adhesion GPCR has been studied via RT-PCR in mouse and rat[50]. More than half of them show predominant expression in the nervous system. In addition, the in-situ hybridization data of several adhesion GPCRs in early zebrafish embryos have been reported[51]. These initial discoveries suggest that the research in the field is still at its infancy stage and more exciting discoveries are still to come.

CONCLUSIONS

By far, we have gained deep structural insights into homophilic or heterophilic interactions between CAMs. It substantially facilitates our understanding of their functions in various biological processes, including gastrulation and brain morphogenesis. The knowledge gained from these studies can guide our studies on novel molecules with similar functional domains and the techniques created for these studies can be further applied to new studies. My research project will focus on the roles of novel adhesion GPCRs in zebrafish gastrulation and brain morphogenesis. Adhesion GPCRs possess unique structural assets. They have diverse functional domains with adhesive properties and the characteristic seven transmembrane region of GPCR. Their enriched expression in the nervous system and early expression in zebrafish embryos indicate their function during gastrulation and brain morphogenesis. Furthermore, *Celsr* and *GPR56* have been shown to play important roles during gastrulation and brain morphogenesis. It again invites investigations on other members of this GPCR family.

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This research article explains how GRAFS classification system for GPCR is formed and why Adhesion GPCR should be an independent family. It serves as the basis of my research hypothesis. This article also offers practical examples of methods used in GPCR bioinformatics, which will be largely applied in the initial step of my research project.
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    By using transplantation method, the authors demonstrated that EphA4MO cells and EphA4-expressing cells sort from each other in rhombomere(r)3 and r5 of mosaic zebrafish embryos. This phenomenon can be well explained by 'differential adhesion hypothesis' (DAH), which is an essential element of my research hypothesis. In addition the transplantation method they used is a preferred method to test DAH in zebrafish embryos.
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FURTHER INFORMATION

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