

Mechanisms of tissue fusion during development

Heather J. Ray and Lee Niswander*

Summary

Tissue fusion events during embryonic development are crucial for the correct formation and function of many organs and tissues, including the heart, neural tube, eyes, face and body wall. During tissue fusion, two opposing tissue components approach one another and integrate to form a continuous tissue; disruption of this process leads to a variety of human birth defects. Genetic studies, together with recent advances in the ability to culture developing tissues, have greatly enriched our knowledge of the mechanisms involved in tissue fusion. This review aims to bring together what is currently known about tissue fusion in several developing mammalian organs and highlights some of the questions that remain to be addressed.

KEY WORDS: Heart, Neural tube, Palate, Tissue fusion

Introduction

During embryonic development, there are many instances when two opposing tissues come into contact and fuse together to form one continuous structure. This type of tissue fusion occurs during the formation of many organs, including the optic cup, palate, heart, neural tube, eyelids and body wall. Superficially, tissue fusion in various organs appears to be similar, and mice deficient in specific transcription factors and signaling molecules exhibit defects in fusion of multiple organs (Zhang et al., 1996; Yu et al., 2010; Pyrgaki et al., 2011). However, organ formation is a complex process that often involves mechanisms highly specific to that tissue, and, accordingly, the precise mechanisms used to drive fusion in individual tissues are also context dependent.

Multiple animal models have been used to study tissue fusion and many types of tissue fusion events occur within a developing organism. Importantly, the inability of tissues to fuse correctly during development can lead to various birth defects, including cleft palate (Abbott, 2010), spina bifida (Copp et al., 1990) and heart defects (Wenink and Zavallos, 1988). The etiology of these debilitating defects in humans is likely to be complex, involving the concurrent disruption of several factors. In model organisms, the ability to ablate molecules specifically in individual tissues or at specific time points has allowed these complex developmental events to be studied and the functions of individual genes to be evaluated. Recent advances in the ability to culture and manipulate developing organs have also identified molecular pathways involved in tissue fusion events. Our understanding of how these fusion events occur has thus grown exponentially over the past 10 years, yet many questions remain.

Here, we summarize what is currently known about tissue fusion in several different organs to highlight both the similarities and differences between these fusion events. In particular, we highlight

studies that largely use mouse models and have provided insights into the molecular and cellular events underlying fusion in the developing heart, palate and neural tube, disruptions of which are responsible for the largest classes of human birth defects. We also outline promising future research avenues that could increase our understanding of these events. For the purpose of this review, we refine our discussion to cover tissue fusion events during which individual cells retain their integrity, rather than cellular fusion events in which multiple cells can fuse to form multinucleated cells, as happens, for example, during skeletal myogenesis.

Tissue fusion during palate development

Palate morphogenesis

The best characterized developmental system, in terms of tissue fusion, is the palate, the tissue that separates the oral cavity from the nasal cavity and forms the roof of the mouth. Palate development is very complex and even small perturbations lead to craniofacial defects known as cleft lip with or without cleft palate (CLP) and cleft palate (CP) (see Box 1). There has long been interest in studying normal palatal formation to understand the etiology behind CLP and CP.

Palate development occurs over an extended period from the time of migration of neural crest cells into presumptive facial mesenchyme [before embryonic day (E) 9.0 in mice] until completion of fusion of the secondary palate (by ~E16), which is one of the last embryonic structures to form by tissue fusion (Johnston and Bronsky, 1995). Interactions between neural crest, facial mesoderm, surrounding endoderm and ectoderm result in five facial prominences by E9.5: the singular frontonasal prominence, paired maxillary prominences and paired

Box 1. Palatal fusion defects

Defects in proper palatal formation lead to a group of craniofacial defects that represent the most common class of birth defects, occurring in 1 to 500 to 1 in 2000 live births. Failure to complete the formation of the primary palate results in cleft lip with or without cleft palate (CLP), while disruptions specifically in secondary palate formation result in cleft palate (CP), with CLP being the most prevalent. Syndromic CLP and CP occur in conjunction with other developmental defects in syndromes such as Van der Woude's and DiGeorge syndrome. Non-syndromic CLP and CP are isolated disorders and account for 70-90% of clefting defects. Genetic linkage studies in families with syndromic CLP and CP have implicated several genes in clefting disorders, including those encoding bone morphogenetic protein 4 (Bmp4), endothelin 1 (Edn1), transforming growth factor α (TGF α), Msx1, poliovirus receptor-related 1 (Pvr1), p63 (Tc1) and interferon response element 6 (Irf6). The importance of several genes identified in mouse models of CP has since been confirmed in individuals with non-syndromic CP and include TGF β 3 and noggin, as well as Msx1, Pvr1 and Irf6. Environmental factors, such as the mother's nutritional status and chemical exposure, can also influence the incidence of CLP and CP. Indeed, there is only a 50% concordance between monozygotic twins and CLP, highlighting a role for non-genetic factors.

HHMI, Department of Pediatrics, Cell Biology Stem Cells and Development Graduate Program, University of Colorado School of Medicine and Children's Hospital Colorado, Aurora, CO 80045, USA.

*Author for correspondence (lee.niswander@ucdenver.edu)

mandibular prominences (Fig. 1A) (Hinrichsen, 1985; Senders et al., 2003). Over the next day, the mandibular processes merge to form the lower lip and jaw. Meanwhile, the frontonasal prominence undergoes a series of morphological changes to appear as two upside down horseshoes, made up of the internal medial and external lateral nasal prominences, which together surround the nasal pits. Prior to initiation of primary palate formation, microvilli line the epithelial surface of the prominences. Just preceding fusion, microvilli disappear from cells at the lower end of the nasal pits. Filopodia then emerge from epithelia of the lateral and medial nasal processes, cross the physical gap between these prominences and anchor in between cells of the opposing tissue, presumably to help initiate tight contact with one another (Millicovsky and Johnston, 1981). Fusion begins at E10.5, first between the maxillary and medial nasal prominences, from posterior to anterior, followed by fusion to the lateral nasal prominences starting at E11. Fusion of these three tissues continues until E12.5 and results in formation of the primary palate, which forms the upper jaw and lip.

Formation of the secondary palate occurs later in development (Farbman, 1968; Hinrichsen, 1985; Griffith and Hay, 1992) (reviewed by Bush and Jiang, 2012). The palatal shelves initially form as vertical outgrowths of the maxillary processes (Fig. 1B). Between E13 and E14, the palatal shelves elevate to lie laterally above the tongue and the medial edges of the shelves come in close proximity of one another. From E14.5 to E16, fusion between the two palatal shelves occurs first in the middle, then progresses both anteriorly and posteriorly. Successful fusion results in formation of the roof of the mouth, or secondary palate, which separates the oral and nasal cavities. Although evidence suggests that the mechanisms of tissue fusion are similar during development of both the primary and secondary palates, the majority of experiments have focused on secondary palate fusion for several reasons. First, secondary palate formation occurs later in embryogenesis; therefore, these fusion events can be disrupted by various teratogens without causing overall embryopathy. Second, secondary palates are comparatively large structures that can be cultured, which makes biochemical manipulation easier. Thus, the majority of work discussed here will center on secondary palate fusion.

Histological changes during palatal fusion

Secondary palate fusion occurs progressively over time; thus, in a single palate multiple steps in fusion of opposing shelves can be visualized. These steps have been termed pre-fusion, fusion and fusion seam steps (Farbman, 1968). The two shelves are each surrounded by medial edge epithelium (MEE), a two- to four-cell thick epithelial layer separated from the mesenchyme by a basal lamina. During pre-fusion, the opposing epithelia are intact with only occasional disruptions in the basal lamina. During fusion, the two epithelia come into contact but the cellular morphology is similar to pre-fusion and there is no evidence of desmosomes, tight junctions or adhesive products between contacting epithelial cells. However, opposing cell layers are tightly adhered as application of physical forces causes cells from one epithelial layer to rip away from the basement membrane and instead remain with the opposing epithelial layer (Farbman, 1968). During the fusion seam step, phagocytes and dying epithelial cells appear in between healthy cells and the basal lamina degenerates (Farbman, 1968). The midline epithelium also thins, owing to convergent extension, as epithelia from both shelves intercalate and form the two-cell layer thick midline epithelial seam (MES). Some MEE cells also migrate away from the midline, where first contact occurs, towards the oral and nasal cavities along the MES to areas termed the 'epithelial triangles' (Martinez-Alvarez et al., 2000). As fusion continues and the epithelial layer is disrupted, mesenchymal cells from both shelves infiltrate the MES to establish continuity between the two tissues (Farbman, 1968; Hinrichsen and Stevens, 1974; Lee et al., 2008).

Apoptosis, epithelial migration and EMT during palatal fusion

Early research discovered evidence of dying cells during palatal fusion, and apoptosis was, therefore, suggested to provide the driving force behind this fusion event (Farbman, 1968; Hinrichsen and Stevens, 1974). Alternatively, Fitchett and Hay proposed that the basal layer of epithelial cells lose their epithelial identity and adopt mesenchymal characteristics (Fitchett and Hay, 1989). Many studies have since attempted to determine the relative contributions of apoptosis versus epithelial-to-mesenchymal transition (EMT) to palatal fusion.

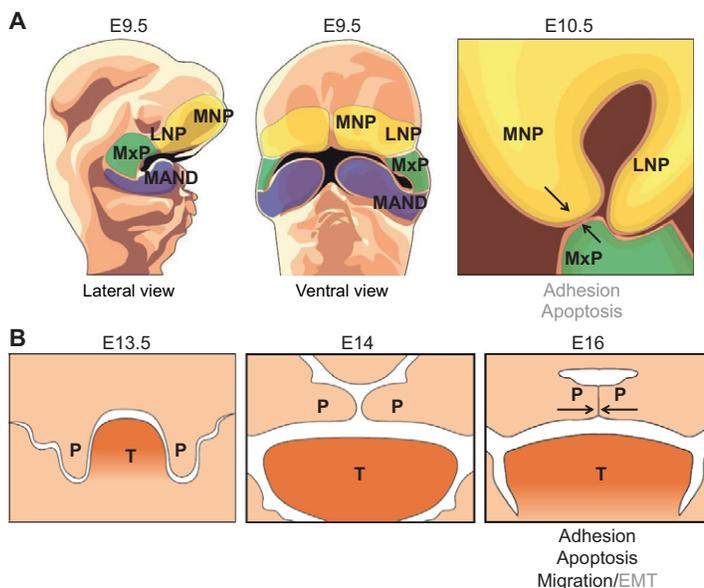


Fig. 1. Tissue fusion during palate development. (A) Fusion during murine primary palate development. At embryonic day 9.5 (E9.5), the frontonasal prominence (yellow) is beginning to develop into the medial nasal prominence (MNP) and the lateral nasal prominence (LNP). Also evident is the maxillary prominence (MxP; green) and the mandibular prominence (MAND; purple). Around embryonic day 10.5 (E10.5), initial tissue fusion occurs between the MNP and the MxP (area between arrows) followed by fusion of the MNP and LNP. (B) Fusion during murine secondary palate development. At E13.5, the palatal shelves (P) are oriented vertically along the tongue (T). By E14, movement of the palatal shelves has brought them into a horizontal position above the tongue. From E14.5 to E16, opposing palatal shelves fuse (area between arrows) to generate the secondary palate. The mechanisms that are known to be necessary (black) or implicated (gray) in primary and secondary palatal fusion are indicated.

Apoptosis, as detected by terminal deoxynucleotidyl nuclear transferase dUTP nick end-labeling (TUNEL), is significantly increased within the MES and epithelial triangles of fusing mouse palates in vivo (Mori et al., 1994). Other studies used lipophilic dyes to trace the fate of MEE cells during secondary palate fusion ex utero (Griffith and Hay, 1992; Sun et al., 2000). Culture of secondary palates isolated prior to palatal fusion showed the dye was sequestered within 'isolation bodies' in epithelial cells, whereas, after further culture to the time of fusion, similar 'isolation bodies' were found within mesenchymal-like cells. In control cultures of single shelves that did not undergo fusion, mesenchymal cells did not contain dye. Thus, it was concluded that contact between palatal shelves initiates EMT in MEE cells. Additional studies have continued to provide evidence for the processes of EMT and apoptosis in palatal fusion (Martinez-Alvarez et al., 2000; Cuervo et al., 2002; Cuervo and Covarrubias, 2004).

However, other studies called into question the importance of apoptosis and/or EMT in palate fusion. In vitro studies using caspase 1 and caspase 3 inhibitors found that secondary palates can completely fuse even in the absence of apoptosis (Takahara et al., 2004). Fate-mapping studies using sonic hedgehog (*Shh*) and keratin 14 (*K14*) promoters to mark MEE cells permanently found no evidence of EMT (Vaziri Sani et al., 2005). So why are there so many studies with disparate results? This issue has been widely discussed and it is thought that variation of in vitro culture techniques and differences between in vitro and in vivo studies could contribute to the discrepancies. In addition, it appears there are differences in how fusion occurs along the anterior to posterior axis of the palatal shelves (Cuervo and Covarrubias, 2004; Takahara et al., 2004; Vaziri Sani et al., 2005). Although controversy remains, evidence points to apoptosis and migration of MEE cells to epithelial triangles as being the main contributors to MES disappearance and tissue fusion, with a possible contribution made by EMT.

Signaling pathways/factors regulating palate fusion

Prior to palatal fusion, the facial prominences and palatal shelves must grow and become precisely positioned to bring opposing tissues in close proximity. Defects in tissue growth, morphogenesis and reciprocal signaling between epithelial and mesenchymal components can lead to CLP and CP. This is observed in mice with mutations in the transcription factors *aristaless-like homeobox 3* (*Alx3*), *Alx4*, *Alx1* (*Cart1*) and activating enhancer binding protein 2 α (*AP2 α* ; *Tfap2a* – Mouse Genome Informatics). Disruption of these factors indirectly results in inability of opposing tissues to fuse, owing to decreased proliferation and/or increased apoptosis causing abnormal positioning of the tissues (Nottoli et al., 1998; Qu et al., 1999; Beverdam et al., 2001). So, although the events regulated by these transcription factors must occur correctly before fusion can occur, to date no transcription factor has been identified that directly drives tissue fusion in the palate.

During their growth and elevation, the palatal shelves are surrounded by a layer of peridermal cells. Periderm acts as a barrier that prevents the palatal epithelium from inappropriately fusing with the tongue and mandible. The transcription factor interferon regulatory factor 6 (*Irf6*) and the Notch ligand jagged 2 (*Jag2*) spatiotemporally maintain the periderm so that the MEE is exposed only at the appropriate time and place for successful palate fusion (Richardson et al., 2009). Mice deficient in *Irf6* or *Jag2* display CP, owing to improper fusion of the palatal shelves with other oral epithelia. *Irf6* and the transcription factor p63 (*Tcp1* – Mouse

Genome Informatics) also regulate epithelial differentiation and apoptosis, misregulation of which interferes with fusion of both the primary and secondary palate (Richardson et al., 2009; Ferretti et al., 2011; Guerrini et al., 2011).

Genetic studies of humans have found that mutations in transforming growth factor (TGF) α and TGF β family members can cause CLP and CP (Holder et al., 1992; Lidral et al., 1997). Mice deficient in *Tgfb3* have fully penetrant CP, providing an animal model with which to study TGF β 3 function in palatal fusion (Proetzel et al., 1995; Koo et al., 2001). As recently reviewed (Iwata et al., 2011), *Tgfb3* is strongly expressed within the MEE at the time of fusion and has many roles, including induction of apoptosis (Martinez-Alvarez et al., 2000) and production of matrix metalloproteases (MMPs) to promote EMT (Blavier et al., 2001). In addition, signaling through the WNT pathway is implicated in palatal fusion, as mice that are homozygous null for two WNT receptors, frizzled 1 (*Fzd1*) and frizzled 2 (*Fzd2*), have completely penetrant CP (Yu et al., 2010). *Wnt11* is expressed in the MEE during fusion and is required for fusion of palatal shelves, as siRNA knockdown of *Wnt11* in vitro results in decreased apoptosis in MEE cells and failure of fusion (Lee et al., 2008). Retinoic acid (RA) signaling in neural-crest-derived mesenchyme also must be tightly controlled, as either too little or too much RA causes CP (Lohnes et al., 1994; Cuervo et al., 2002). Finally, the secreted ECM protein periostin is produced by mesenchymal cells directly under the MEE and induces EMT in epithelial cells within the MES, although periostin has not been shown to regulate palatal fusion directly (Kitase et al., 2011).

Ephrin signaling is also important in secondary palate fusion. Ephrins are cell surface ligands that bind to ephrin receptor tyrosine kinases (Eph) on opposing cells. Upon binding, signaling is initiated in both cells via reciprocal signaling from the ligand (reverse signaling) and receptor (forward signaling). Mice deficient in both neural kinase (*Nuk*; *Ephb2* – Mouse Genome Informatics) and *Sek4* (*Ephb3* – Mouse Genome Informatics), two ephrin receptors expressed along the MES, have highly penetrant CP (Orioli et al., 1996). Forward signaling through *Ephb2* and *Ephb3* is required for palatal fusion; however, these signaling molecules regulate proliferation and not palatal fusion itself (Risley et al., 2009). Reverse signaling through ephrin B2, however, directly affects palatal shelf fusion, and ephrin B2 is expressed at high levels in MEE cells just prior to fusion and within the MES and neighboring mesenchyme during fusion. This reverse signaling is PI3 kinase dependent and may involve interaction with claudins, which are important epithelial adhesion proteins (Dravis and Henkemeyer, 2011; San Miguel et al., 2011).

Thus, through histological, genetic and culture experiments, there is a good understanding of the mechanisms underlying secondary palate fusion, and to a lesser extent fusion of the nasal and maxillary prominences to form the primary palate.

Tissue fusion in the neural tube

Neural tube morphogenesis

One of the earliest embryonic structures to form is the neural tube (NT), which gives rise to the central nervous system. Initially, ectoderm along the dorsal side of the embryo is specified to be neuroepithelium and this epithelium then thickens to form the neural plate (Fig. 2A). In response to signals between the neuroepithelium and surrounding tissues, and the forces generated by tissue movements, the neuroepithelium forms hinge points and bends on both sides in a U shape to elevate the neural folds (Fig. 2B). The opposing folds approach one another (Fig. 2C) and then

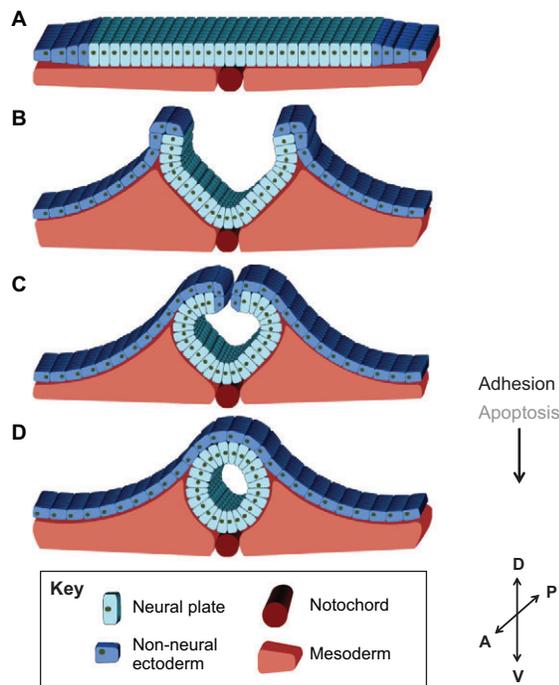


Fig. 2. Development and fusion of the neural tube. (A) Thickening of the dorsal neuroepithelium results in formation of the neural plate. (B) The neuroepithelium bends dorsally to form the neural folds. (C) Further bending brings the neural folds in close opposition to each other. Non-neural ectoderm cells cover the edge of the neuroepithelium. (D) Separation of neural and non-neural ectoderm and fusion of these tissues results in formation of the closed neural tube covered by a sheet of ectoderm. Necessary (black lettering) and observed but not required (gray lettering) mechanisms of neural tube fusion are indicated. D, dorsal; V, ventral; A, anterior; P, posterior.

come into contact to undergo a tissue fusion event that results in formation of the continuous NT (Fig. 2D). During NT fusion, the neuroepithelium separates from the neighboring non-neural ectoderm and then both tissues close to form the neural tube covered by a single sheet of non-neural ectoderm. Failures in neural tube formation lead to a class of birth defects collectively referred to as neural tube defects (NTDs) (see Box 2).

There are over 240 mouse models of NTDs (Harris and Juriloff, 2010). Many are due to recessive mutations of individual genes, with some showing low penetrance NTDs when haploinsufficient. Genetic complexity is also demonstrated in models of compound mutations of two or three genes. The majority of these mouse models exhibit exencephaly, whereas spina bifida alone is less common. Although NTDs are ultimately due to failure of NT closure, very few mouse models directly relate to fusion, the final step of NT closure. For example, mice mutant for genes in the planar cell polarity (PCP) pathway, such as *Vangl2* and *Intu*, exhibit NTDs due to lack of convergent extension, which is needed to elongate the neural plate and bring the neural folds close together for fusion to occur (Ybot-Gonzalez et al., 2007; Wansleben et al., 2010; Zeng et al., 2010). Moreover, many NTDs disrupt proliferation, hinge point formation, primary ciliogenesis or neural patterning, but these important processes occur independently of NT fusion (Murdoch and Copp, 2010).

Histological studies of neural tube fusion

Studies of NT fusion in the head regions of mice using transmission electron microscopy (TEM) revealed that the tissue layer that makes initial contact differs depending on the region of closure (Geelen and Langman, 1979). At the rhombencephalon and mesencephalon, non-neural epithelial cells first come into contact followed by the neural epithelium. In the anterior neuropore, the neural epithelium appears to make the first contact. In the prosencephalon region, both cell layers appear to make contact simultaneously. However, no matter which tissue layer first comes into contact, the cells exhibit similar behaviors at the time of fusion along the rostrocaudal axis; finger-like projections extend from cells of both neural folds and intercalate with each other as the two folds come together. No epithelial junctional complexes are observed except in the mesencephalon, where junctions are observed between cells of the non-neural ectoderm. Recent advances in mouse embryo ex utero culture techniques have allowed for live imaging of NT closure and hence a more dynamic evaluation of cellular behaviors during this process. In such a study of cranial NT closure, cellular projections were also seen in the gap between the neural folds (Pyrgaki et al., 2010).

Transcriptional regulation of neural tube fusion

Recent genetic studies provide important information on the molecular mechanisms that directly regulate fusion of the neural folds. Two members of the Grainyhead-like (*Grhl*) transcription factor family, *Grhl2* and *Grhl3*, are expressed in the non-neural ectoderm during NT fusion in discrete, as well as overlapping, patterns; loss of expression of one or both genes leads to NTD and developmental defects of many tissues (Rifat et al., 2010). Here, we concentrate on NTDs; however, *Grhl2* mutants, for example, also show failure of face and body wall closure, suggesting that *Grhl2* plays a more general role in multiple fusion processes, although this has not been directly tested. Different alleles of *Grhl2* show some phenotypic differences, possibly owing to differences in genetic background and the specific mutation, yet all exhibit highly penetrant exencephaly and cleft face, and some alleles show spina bifida (Rifat et al., 2010; Werth et al., 2010; Pyrgaki et al., 2011). Histological analysis of *Grhl2* mutants at E9.5 showed proper elevation and apposition of the neural folds in the head region but fusion itself failed to occur (Pyrgaki et al., 2011). *Grhl2* directly regulates expression of E-cadherin and claudin 4, both of which are important for formation of epithelial junctional complexes, and *Grhl2* mutant mice show decreased expression of these two proteins in non-neural ectoderm with a concurrent increase of N-cadherin (Werth et al., 2010). E-cadherin has been implicated in NT closure; knockdown of E-cadherin with antisense oligonucleotides during NT closure in rats resulted in NTD (Chen and Hales, 1995). Additional direct and indirect *Grhl2* targets include molecules involved in adhesion, such as desmoglein 2, desmocollin 2, desmoplakin and epithelial cell adhesion molecule, and an increase in the matrix metalloproteases *Adamts1* and *Adamts3* (Pyrgaki et al., 2011). The identification of a number of adhesion genes regulated by *Grhl2*, and perhaps *Grhl3*, presents an opportunity for further study of the role of adhesion in NT fusion.

Loss of *Grhl3* function also results in fully penetrant NTDs, characterized by spina bifida or curled tail, and infrequent exencephaly (Ting et al., 2003). *Grhl3* can activate transcription and several epithelial-specific genes are direct targets of *Grhl3*, including keratin 5, keratin 6 and keratin 10, filaggrin and involucrin (Boglev et al., 2011). *Grhl2* and *Grhl3* have overlapping expression patterns and embryos with compound heterozygous and

homozygous null mutations of both *Grhl2* and *Grhl3* have demonstrated their relative contribution to NT closure: closure point 3 appears to depend on *Grhl2*, whereas closure point 2 is regulated by both transcription factors with the overall gene dose being the most important factor in successful closure. However, closure point 1 occurs in the absence of both *Grhl2* and *Grhl3* (Rifat et al., 2010). A role for *Grhl3* in hindgut endoderm has been suggested in spinal NT closure, as loss of *Grhl3* expression in the hindgut endoderm leads to decreased proliferation, which causes increased ventral curvature and hence pulls the neural folds apart so that fusion cannot take place (Ting et al., 2003; Gustavsson et al., 2007).

Two well-characterized mouse NTD models that arose from spontaneous mutations are axial defects (*Axd*) and curly tail (*Ct*). In both cases, heterozygous embryos exhibit a 'curly tail' due to delayed closure of the posterior neuropore (PNP), whereas homozygotes display spina bifida. The causative mutations are not known but both result in misregulation of *Grhl* expression. *Axd* mutants exhibit increased *Grhl2* expression, and NT closure can be restored by decreasing the level of *Grhl2* (Brouns et al., 2011). *Ct* mutants show decreased expression of *Grhl3*, and NT closure can be rescued by *Grhl3* overexpression (Gustavsson et al., 2007). Thus, these studies suggest that *Grhl* activity must be tightly controlled during NT closure.

Although early EM studies indicated a lack of epithelial junctional complexes at the NT fusion seam (Geelen and Langman, 1979), the studies above highlight a role for cell adhesion in NT fusion. Dynamic regulation of cell-cell adhesion during NT fusion is further implicated by studies of the tumor suppressor gene neurofibromatosis type 2 (*Nf2*) (McLaughlin et al., 2007). *Nf2* (also known as merlin) regulates assembly of apicolateral junctional complexes. *Nf2* expression decreases at the tips of the dorsal neural folds just prior to fusion, and then sharply increases after completion of fusion. Knock out of *Nf2* in the neuroepithelium at E8.5 does not affect the initial fusion process but, after E9.5, the NT reopens, resulting in NTDs. Neural and non-neural ectoderm cells are healthy in *Nf2* mutants but they detach from the apical surface due to a lack of epithelial junctions. Interestingly, *Nf2* mutants have other developmental defects, including cleft palate, eye and body wall defects and cardiac ventricle septal defects, although whether these represent fusion defects remains to be tested (McLaughlin et al., 2007). Thus, *Nf2* may act to differentially regulate cell-cell adhesion during multiple tissue fusion events.

The transcription factor AP-2 α is also required for NT closure and proper palate formation (Schorle et al., 1996; Zhang et al., 1996). In AP-2 α mutant embryos, the neural folds elevate but fusion does not occur. However, the failure of neural fold fusion may result from overproliferation of the neuroepithelium such that the folds cannot physically meet. Loss of the transcriptional activators CBP (Crebbp – Mouse Genome Informatics) and p300 (Ep300 – Mouse Genome Informatics), as well as their co-activators Cited2 and Cart1 (Alx1 – Mouse Genome Informatics), all individually result in many developmental defects, including NTDs. All of these proteins are found specifically in the dorsal neural folds at the time of NT closure, suggesting they may regulate neural fold fusion (Bhattacharjee et al., 2009).

Signaling pathways/factors involved in neural tube fusion

Similar to palate fusion, signaling via ephrins and their receptors is required for NT fusion. Ephrin A5 (*Efna5* – Mouse Genome Informatics) and the *Epha7* receptor are expressed at the tips of the

Box. 2. Neural tube defects

Failure to close the neural tube during early development leads to a class of birth defects collectively referred to as neural tube defects (NTDs), which occur in roughly 1 in every 1000 live births worldwide. In mice, initial contact between the neural folds occurs in three places along the rostral-caudal axis, but there is debate as to the number of initial contact points in human embryos. Studies in mice have shown that failure of fusion at closure point 1 (the hindbrain/cervical boundary) leads to craniorachischisis, while failure of closure at point 3 (the rostral end of forebrain) or point 2 (the forebrain/midbrain boundary) causes a cranial NTD called anencephaly in humans or exencephaly in mice. The caudal end of the neural tube closes last at the posterior neuropore (PNP) and failure of PNP closure causes spina bifida, the most common human NTD.

Little is known about the genetic basis for NTDs in humans, although it is thought that both genetic and environmental factors play a role. NTDs are also often found associated with other developmental disorders, such as trisomy 13, trisomy 18 and some chromosomal rearrangements, which has led to the hypothesis that proper gene dose is crucial for closure of the neural tube. NTDs are also sometimes associated with the ciliopathy disorder Meckel-Gruber syndrome, and recent genetic studies have identified mutations in the planar cell polarity gene Van Gogh like 2 (*VANGL2*) in humans with spina bifida. An additional gene implicated in defective neural tube closure in humans is platelet-derived growth factor receptor A (*PDGFRA*) as a recent study found that some *PDGFRA* promoter haplotypes are associated with increased risk for NTDs. In general, the relative paucity of individual gene associations with human NTDs and the low incidence of NTD reoccurrence in families with one NTD pregnancy suggests a complex and multifactorial etiology, and has led to the belief that more global changes in gene expression could be responsible for development of this class of birth defects.

cranial neural folds. Mice deficient in *Efna5* or *Epha7* exhibit exencephaly at low penetrance, apparently owing to an inability of the neural folds to fuse (Holmberg et al., 2000). In addition, *Efna1* and *Efna3* are expressed in the caudal neural folds and PNP, as are the receptors *Epha1*, *Epha2*, *Epha4* and *Epha5*. Both *Epha2* and *Epha4* are strongly expressed at the tips of the neural folds during fusion with *Epha2* expressed in non-neural ectoderm. Blocking EphA activity, with an EphA fusion protein to disrupt ligand-receptor interactions, in the caudal region resulted in increased PNP size, which was attributed to defective tissue fusion and not to a problem in NT morphogenesis (Abdul-Aziz et al., 2009).

Two recent studies discovered roles for G-protein-coupled receptor (GPCR) signaling in NT fusion. The spontaneous mouse mutation *vacuolated lens* (*vl*), which is considered a model of failure of neural fold fusion, results from mutation of an orphan GPCR, *Gpr161*, which is expressed in the neural folds (Matteson et al., 2008). Another class of GPCRs is the protease-activated receptors (PARs), which respond to proteases in the environment. Compound null mutations for *Par1* and *Par2* (*Gpr172b* – Mouse Genome Informatics) result in exencephaly, and occasional spina bifida or curly tail. *Par2* is expressed in non-neural ectoderm at the time of fusion and disruption of downstream signaling (G_i or $Rac1$) in the non-neural ectoderm also causes exencephaly (Camerer et al., 2010).

Apoptosis during neural tube fusion

In contrast to the requirement for apoptosis in primary and secondary palate fusion, a role for apoptosis during NT fusion is unclear. Scanning electron microscopy (SEM) showed scattered

apoptotic cells in neural tissue undergoing fusion (Geelen and Langman, 1979). TUNEL staining showed a correlation between the presence of apoptotic cells and the bending and fusing of opposing neural folds (Massa et al., 2009). To more directly investigate the role of apoptosis in NT closure, *in vivo* and *in vitro* models have been used. *Casp3^{-/-}* or *Apaf1^{-/-}* embryos fail to close the caudal midbrain and hindbrain regions (Cecconi et al., 1998; Leonard et al., 2002; Massa et al., 2009). Although the absence of apoptosis in these mice leads to cranial NTDs, it is difficult to draw conclusions about the direct role of apoptosis in fusion owing to the general effects that a lack of apoptosis may have on other cellular processes. In embryo cultures in which pharmacological inhibitors of apoptosis were added just at the time of NT closure, it was found that apoptosis is not required for NT closure (Massa et al., 2009). A recent study used live imaging techniques to further explore a role for apoptosis in NT closure (Yamaguchi et al., 2011). In *Casp3^{-/-}* or *Apaf1^{-/-}* mice, there was reduced bending of the neural plate and reduced flipping of dorsal ridges in the midbrain-hindbrain region. Additionally, these mutant mice and wild-type embryos treated with caspase inhibitor showed reduced speed of closure at closure points 1 and 2, although complete closure could still occur. This work suggests a model in which apoptosis helps NT closure proceed within a necessary time frame before additional forces prohibit successful fusion.

Tissue fusion in the developing heart

Heart morphogenesis

Tissue fusion plays an integral role during development of the mammalian heart, as it transforms a simple tube-like structure into a complex four-chambered organ. Fusion occurs in several different areas of the heart and is involved in separation of the atria and ventricles, and in division of the initial singular outflow tract into the aorta and pulmonary trunk. There is considerable variation in the processes of fusion within the heart, possibly owing in part to the contribution of neural crest cells in the developing outflow tract (Poelmann et al., 1998; Waldo et al., 1998). Early understanding of the morphological and fusion events in the developing heart came from histological and SEM studies. Moreover, direct comparisons of this morphogenetic process have been made between mouse, chicken and human embryonic hearts (Pexieder, 1978; Thompson et al., 1985; Vuillemin and Pexieder, 1989). Unfortunately, the advances in *in vitro* organ culture methods that spurred a mechanistic understanding of palate and neural tube fusion have lagged behind in the field of heart development. Thus, of the three organs highlighted in this review, we know the least about what drives fusion in the heart.

By E9.5 in the mouse, the unseptated tubular heart has already looped to the right (Webb et al., 2003). Between E9.5 and E10.5, two sets of endocardial cushions (ECs) begin to grow towards each other across the open internal space of the tube (Fig. 3A). The first set, the conotruncal ECs, form across the common outflow tract (the conotruncus; CT), which leads outwards from the primitive right ventricle to the aortic sac. The conotruncal ECs are further divided into proximal conotruncal and distal conotruncal EC pairs (PCEC and DCEC). Eventual fusion of the DCECs creates the conotruncal septum, which separates the aortic and pulmonary trunks between E12.5 and E13.5. The PCECs fuse later and contribute to the membranous ventricular septum. The second set of cushions, the atrioventricular ECs, develop across the bend of the looped heart within the atrioventricular canal and eventually fuse to separate the atrial and ventricular spaces (Webb et al., 1998). At the same time, the primary atrial septum and the

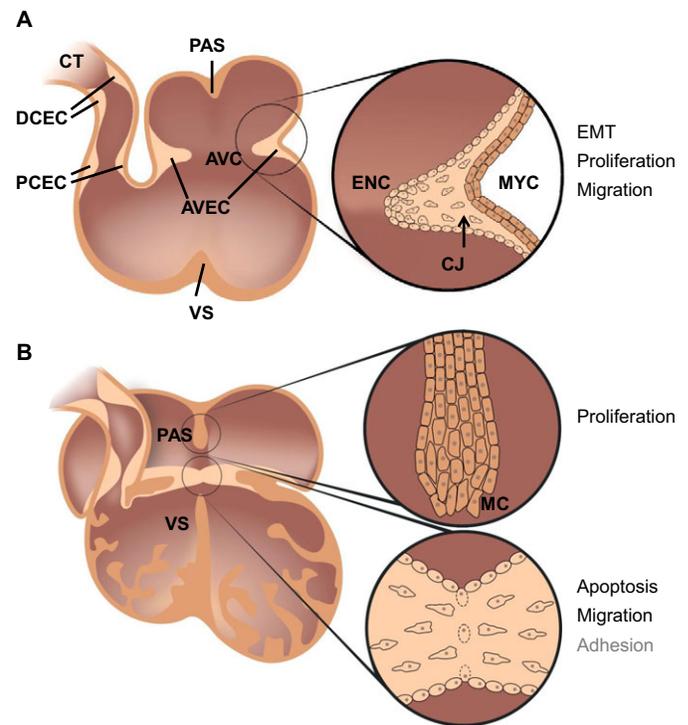


Fig. 3. Fusion during development of the heart. (A) Between E9.5 and E10.5, endocardial cushions (ECs) begin to form across the conotruncus (CT) and the atrioventricular canal (AVC): the proximal conotruncal endocardial cushions (PCECs), the distal conotruncal endocardial cushions (DCECs) and the atrioventricular endocardial cushions (AVECs). The inset provides a closer view of AVEC formation. Endocardial cells (ENC) proliferate, undergo an epithelial-to-mesenchymal transition and migrate into the cardiac jelly (CJ), which is produced by myocardial cells (MYC). (B) Between E12.5 and E13.5, the ECs are in close opposition and begin to fuse. The myocardium-derived primary atrial septum (PAS) and ventricular septum (VS) grow inwards towards the AVEC. The top inset illustrates the mesenchymal cap (MC) that is present at the growing end of both the PAS and the VS. The bottom inset illustrates the fusing ECs as the two cushions come into contact: endothelial integrity at the midline breaks down and the mesenchymal cells migrate across the midline to form 'mesenchymal bridges'. Necessary (black lettering) and possible (gray lettering) mechanisms involved in EC fusion are indicated.

ventricular septum begin to grow inwards from the roof of the common atrium and floor of the ventricular chamber, respectively. These two septa will fuse with the atrioventricular ECs to create the four chambers of the heart (Fig. 3B). These fusion events occur in a precisely coordinated pattern between E12.5 and E13.5. Once fusion occurs, myocardiocytes invade the ECs and tissue remodeling creates the major valves of the heart. Thus, defects in fusion result in a multitude of cardiac defects (see Box 3).

The primary heart tube consists of an external layer of myocardium and an internal endocardial layer. These layers are separated by extracellular matrix called cardiac jelly produced by the myocardium (Henderson and Copp, 1998). As the endocardial cushions develop, endocardial cells proliferate, undergo EMT and migrate into the cardiac jelly (Fig. 3A, inset). As the two cushions come into contact, the endocardial cell barrier breaks down, and mesenchymal cells form a bridge between the two cushions to stabilize fusion of the two tissues (Fig. 3B; bottom inset). If bridge

formation is perturbed, then fusion is incomplete and the cushions are pulled apart (Ghyselinck et al., 1998). The atrial and ventricular septa, by contrast, both consist of growing myocardium with leading edges of mesenchymal cells. These mesenchymal ‘caps’ then fuse with mesenchymal cells of the endocardial cushions (Fig. 3B, insets).

Signaling pathways/factors involved in fusion during heart development

Signaling between endothelial and mesenchymal components is important for regulating growth of the ECs, and perturbation of several signaling molecules can lead to fusion defects. Maternal deficiencies in retinoic acid (RA) cause many congenital abnormalities, which are together termed vitamin A deficiency syndrome, that affect several tissues, including the heart. RA signals through heterodimers of RA receptors and retinoic X receptors (RXRs). Mice deficient in both *Rxra* and *Rxb* exhibit defects in conotruncal EC fusion, leading to ventricular septal defects (Ghyselinck et al., 1998). In these mutants, EC contact occurs but the endocardium does not break down and mesenchymal bridge formation is also perturbed owing to increased apoptosis and decreased mitosis of EC mesenchyme. This fusion defect is specific to the conotruncus, whereas atrioventricular EC fusion proceeds normally.

Conversely, deficiencies in ephrin signaling lead to fusion defects specifically in the atrioventricular ECs and not in the CT (Stephen et al., 2007). *Epha3* is expressed in the mesenchyme of both the atrioventricular and conotruncal ECs, and in the mesenchymal cap of the atrial septum, whereas *Epha1* is expressed in the adjacent endothelial cells. *Epha3*^{-/-} mutants exhibit defects in EMT and mesenchymal cell migration, and show increased apoptosis in both sets of ECs, with delayed fusion of the atrioventricular ECs, as well as defective atrial septum formation.

Versican (also known as PG-M) is a chondroitin sulfate proteoglycan expressed in areas of EMT in mouse embryos, although it is considered non-permissive for migration and instead is implicated in regulating cell-cell or cell-substratum adhesion. In the heart, versican is dynamically expressed in the developing ventricles, conotruncus and trabeculated myocardium, and probably plays several different roles. Moreover, versican is highly expressed in the mesenchymal cap of both the atrial and ventricular septa and its expression sharply declines following EC fusion. Versican is also highly expressed within the growing ECs, and in versican knockout mice, the ECs do not grow and endocardial cells show defective migration in tissue explants (Henderson and Copp, 1998). A similar phenotype is seen in mice deficient for hyaluronin, another ECM component of the cardiac jelly (Camenisch et al., 2001). Thus, these ECM components are required for development of the ECs and septa; however, a specific involvement in fusion of these tissue components has yet to be investigated.

Another ECM component expressed in the developing heart is the secreted matricellular protein cysteine-rich angiogenic inducer 61 (Ccn1; Cyr61 – Mouse Genome Informatics). Ccn1 interacts with integrin receptors to promote adhesion, migration, proliferation, differentiation, survival or death, depending on the cell type. Ccn1 is expressed in the space between the septa and cushions prior to fusion, and *Ccn1* knockout mice exhibit fully penetrant atrioventricular or valvular septal defects owing to lack of tissue fusion. Ccn1 may promote fusion through induction of matrix metalloproteinase 2 (Mmp2), which contributes to tissue remodeling, and by promoting cell survival (Mo and Lau, 2006).

Box 3. Fusion-related cardiac defects

During development of the heart, growth and fusion of the endocardial cushions (ECs), primary atrial septum and ventricular septum are key processes in formation of the four chambers and separation of the aorta and pulmonary trunk. After successful fusion of these tissues, remodeling occurs such that the cushions develop into the definitive septa and valves that regulate blood flow through the heart. Failures in proper formation and fusion of these tissues result in malformations of the outflow tract, such as double outlet right ventricle, and a multitude of valvular defects that account for about 7.5% of congenital heart disease in humans. Additionally, ventricular septal defects represent the most common form of human cardiac birth defects. These heart defects disrupt overall blood flow, resulting in a left to right shunting of the blood and volume overload of both ventricles that eventually leads to heart failure early in life.

Although some defects probably arise from specific errors in EC and septal fusion, more is known about the defects that occur due to disruptions in tissue growth and positioning. In fact, mutations in genes encoding transcription factors that regulate heart morphogenesis, such as GATA-binding protein 4 (*Gata4*), Zic family member 3 (*Zic3*), NK2 homeobox 5 (*Nkx2.5*) and T-box protein 5 (*Tbx5*), have been identified in humans with a variety of septal defects. Additionally, defects in neural crest cell migration and retinoic acid signaling lead to outflow tract defects. However, further research is necessary to define the molecular mechanisms underlying EC and septal fusion, and to determine the extent to which disruptions to these mechanisms can contribute directly to heart defects.

Neural crest cells, which contribute to the outflow tract of the developing heart, are also required for conotruncal EC fusion; however, the mechanisms by which they influence fusion may be different between the distal and proximal cushions. These cells migrate through the third pharyngeal arch to the conotruncal ECs between E9.5 and E12.5. Mice deficient in neural crest-derived meltrin β , an ADAM family metalloprotease, exhibit normal fusion of the distal ECs, but are unable to fuse the proximal ECs, even though they come in close proximity (Komatsu et al., 2007).

As with the palate and neural tube, WNT signaling appears to be involved, as evidenced by ventricular septal defects in *Fzd1/Fzd2* knockout mice. However, this could result from general deficiencies in neural crest migration and direct involvement with heart tissue fusion remains to be determined (Yu et al., 2010). A number of transcription factors are also needed for correct formation of the heart (Takeuchi et al., 2003); however, none of these has yet been implicated specifically in fusion.

Apoptosis during tissue fusion in the heart

Conserved patterns of cell death have been observed in chick, rat and human embryonic hearts within the fusion seams of both the outflow tract and atrioventricular ECs, and in the ventricular septum (Pexieder, 1975). These observations, along with teratogenic studies using chick hearts, led Pexieder to hypothesize that cell death plays an important role in heart morphogenesis. TUNEL assays also demonstrated dynamic patterns of apoptosis in the heart from E11.5 onwards (Zhao and Rivkees, 2000). At E12.5, when the atrioventricular ECs start to fuse, apoptosis occurs within the fusion seam, then decreases after fusion is complete. Fusion of these cushions occurs in a zipper manner from anterior to posterior, and the dynamic increase in apoptosis closely follows this pattern. However, in the conotruncal ECs, increased apoptosis is seen not only in the fusion seam, but throughout the cushions, and therefore may play a more general role in tissue remodeling

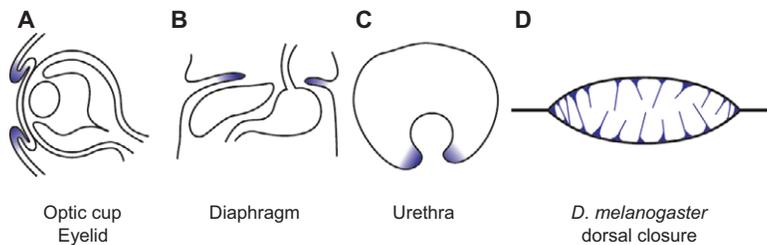


Fig. 4. Additional tissue fusion events during development. (A) During development of the eye, two tissue fusion events occur: the first drives formation of the optic cup; the second fuses the eyelids together. (B) Tissue fusion occurs to generate the mammalian diaphragm. (C) Opposing epithelia fuse to complete the tubular urethra. (D) In *Drosophila melanogaster*, fillipodia (blue spikes) extend from opposing epithelia to aid in the dorsal closure fusion event. Tissue fusion events occur between tissues shaded in blue. Adapted, with permission, from Yu et al. (Yu et al., 2010).

(Zhao and Rivkees, 2000). A subset of neural crest cells within the conotruncal ECs is highly susceptible to apoptosis, and this may be reflected in the differential pattern of apoptosis observed (Poelmann et al., 1998). These studies suggest a role for apoptosis in fusion events of the heart; however, additional research is needed to determine its necessity, as has been carried out in the palate and NT.

Tissue fusion in other developmental contexts

So far, we have focused on tissue fusion events in the murine palate, neural tube and heart. There are, however, many more fusion events that occur during development of mammals and other organisms. As shown in Fig. 4, proper development of the eyes, diaphragm and urethra all require tissue fusion. Failures of these fusion events result in birth defects such as coloboma, diaphragmatic hernia and hypospadias, respectively. Very little is known about the mechanisms of fusion in these tissues. During urethral development, disruptions in Shh and Fgf signaling lead to decreased outgrowth of the urethra and hypospadias, although fusion itself is not disrupted (Cohn, 2011). Signaling through several Ephs is also involved in urethral closure; however, there have been no direct mechanisms identified that drive this tissue fusion event (Shaut et al., 2007; Cohn, 2011). It remains to be seen whether there are highly conserved mechanisms among some or all of the tissue fusion events that occur during development.

Common and divergent mechanisms for tissue fusion

Superficially, many of the tissue fusion events discussed above appear similar. Prior to fusion, tissues must proliferate and position themselves such that they are in apposition at the right time and place for fusion to occur. During fusion, two separate tissues must come together to form one continuous tissue. However, detailed studies have shown that while some mechanisms are conserved among multiple tissues, there are also mechanisms that are highly specific to individual tissues. The issue remains of whether knowledge gained from studies in the palate, heart and neural tube

can be used to understand the mechanisms behind fusion in less studied tissues. There are many human syndromes that are associated with multiple fusion defects, and genetic studies may help to uncover common mechanisms that control tissue fusion. Most of what we currently know about these syndromes, however, cannot be directly attributed to tissue fusion but rather to more global changes in key developmental signaling pathways or changes in gene dose. For example, Pallister-Killian syndrome causes cleft palate and diaphragmatic hernia, owing to an abnormal extra isochromosome (12p) (Holder et al., 2007). It may be more useful to infer similarities in fusion mechanisms based on the germ layers involved. For example, NT and urethral fusion occurs between opposing ectodermal cells, whereas fusion in the conotruncus, primary palate and diaphragm involves neural crest derivatives. Thus, there may be germ layer-specific mechanisms. There are also similarities in tissue fusion events among different species. Neural tube fusion also occurs in frog, chick and zebrafish embryos, whereas a similar event known as dorsal closure occurs in *Drosophila*. These organisms can provide distinct experimental and genetic advantages for studying tissue fusion. Indeed, insights derived from one organism have later been confirmed in mice. For example, Irf6 function in palate fusion is similar in chick and mice (Knight et al., 2006). Nonetheless, a common function is not always the case and it is important to not draw conclusions without careful analysis between animal models. In the case of NT closure, studies in chick found that apoptosis is indispensable for fusion, whereas, in mice, apoptosis plays a minimal role in NT fusion (Weil et al., 1997; Massa et al., 2009; Yamaguchi et al., 2011). *Drosophila* dorsal closure differs from NT closure in that the dorsal epithelia move along the amnioserosa rather than meeting across a physical gap (Jankovics and Brunner, 2006). Yet in both species fillipodia are extended towards each other before fusion occurs; in *Drosophila* this is required for zipping the epithelial sheets together. Therefore, although tissue fusion events may use

Table 1. Mechanisms of tissue fusion

| | Palate | | Neural tube | Heart | |
|-----------|----------------------------|---|---|---|--------------------------|
| | Primary | Secondary | | Conotruncal ECs | Atrioventricular ECs |
| Adhesion | Yes | Yes | Yes | Yes | Yes |
| Apoptosis | Yes | Yes | May be required | May be required | May be required |
| EMT | ? | May be required | No | Yes | Yes |
| Migration | ? | Yes | No | Yes | Yes |
| Signaling | TGF α , TGF β | TGF α , TGF β , ephrin B2, EphB3 Wnt11, Fzd1, Fzd2 | Ephrin A, EphA family members, Gpr161, Par1, Par2 | RXR α , RAR β , meltrin β | Ephrin A1, EphA3 Ccn1 |

ECs, endocardial cushions; EMT, epithelial-to-mesenchymal transition; RAR, retinoic acid receptor; RXR, retinoic X receptor; TGF, transforming growth factor.

somewhat different strategies or genes, information gained from the study of one fusion event could be extremely useful in learning about other fusion events.

Conclusions

This review of tissue fusion events in the developing mammalian palate, neural tube and heart highlights several key processes that they have in common, as well as processes that are different or yet unexplored (summarized in Table 1). For two separate tissues to become one cohesive entity, cells in each tissue must decrease their tight associations with one another in order to allow new contacts to form with cells from the opposing tissue. Although this general principle holds true in the tissues discussed here, the process by which it is achieved varies from intercalation of opposing epithelial cells, to EMT, to apoptosis or a combination of these processes. Apoptosis is observed during fusion of all three tissues but its importance in neural tube and heart fusion remains unclear. Changes in cell-cell adhesion will underlie fusion, but a molecular understanding of the regulation of cell adhesion during fusion is still limited. One clear similarity is the requirement for ephrin/Eph signaling. Ephrin/Eph signaling is usually associated with repulsion but in the three organs discussed above, ephrin/Eph signaling elicits an adhesive response, the loss of which results in failure of tissue fusion. Grhl family members transcriptionally regulate adhesion genes and are required for fusion in the neural tube and face, and probably the body wall and eye; however, a possible role in heart fusion is unexplored.

Although we are beginning to understand the mechanisms that drive tissue fusion in embryogenesis, many questions remain. Communication between opposing tissues is likely to be crucially important but has as yet been little studied. During neural tube fusion, PARs are expressed specifically within the non-neural ectoderm, suggesting that proteases in the paracellular environment could activate these receptors. But from where would such secreted factors arise? Do cells from the opposing tissues secrete factors that aid the two tissues in finding each other across open space? Tight coordination of cell proliferation is also a key factor in correct alignment of opposing tissues such that fusion can occur. However, what triggers the cellular responses that arise before the tissues come into contact (such as the filopodia that epithelial cells extend towards each other)?

All of the fusion events described here are tightly regulated such that even small perturbations can disrupt tissue fusion. However, a great challenge when studying tissue fusion using an animal model is that the processes preceding tissue fusion are also highly complex and disruptions to these earlier events can result in disease phenotypes that resemble fusion defects, even though fusion may not be directly affected. The ability to culture palatal shelves in vitro provides a way to uncouple the final tissue fusion from earlier events; however, there are no similar protocols for specifically analyzing tissue fusion in the heart and NT. Historically, most studies of these tissues have relied on static images obtained from embryos at various stages, leading sometimes to ambiguous results. Recent advances in in vitro culture techniques of whole embryos and live imaging techniques, along with the use of tissue-specific fluorescent reporters and gene disruptions, can provide a dynamic analysis of tissue morphogenesis. This will enable more detailed evaluation of specific mechanisms of tissue fusion and address how specific genetic changes affect the cell behaviors necessary for tissue fusion.

It is unclear whether knowledge gained from study of palate, neural tube and heart fusion can be extrapolated to other tissue fusion events, such as during development of the body wall and eye. A few genes regulate fusion in more than one tissue, but the large number of cellular and molecular differences outlined here suggests there may not be a unifying mechanism. Finally, our understanding of the mechanisms involved in embryonic tissue fusion events largely comes from single gene mutations in animal models. Although individual mutations have been identified in humans with developmental defects, the cause of most birth defects is likely to be more complex. The biggest challenge then remaining is to elucidate and link the complex interplay between genetic factors, as well as environmental influences, to the etiology of tissue fusion defects in the developing human embryo.

Acknowledgements

We are grateful to LeAnn Fenton for the figures and to Trevor Williams for critical reading of the manuscript.

Funding

This work was supported by the National Institutes of Health. L.N. is an Investigator of the Howard Hughes Medical Institute. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

References

- Abbott, B. D.** (2010). The etiology of cleft palate: a 50-year search for mechanistic and molecular understanding. *Birth Defects Res. B Dev. Reprod. Toxicol.* **89**, 266-274.
- Abdul-Aziz, N. M., Turmaine, M., Greene, N. D. and Copp, A. J.** (2009). EphrinA-EphA receptor interactions in mouse spinal neurulation: implications for neural fold fusion. *Int. J. Dev. Biol.* **53**, 559-568.
- Beverdam, A., Brouwer, A., Reijnen, M., Korving, J. and Meijlink, F.** (2001). Severe nasal clefting and abnormal embryonic apoptosis in *Alx3/Alx4* double mutant mice. *Development* **128**, 3975-3986.
- Bhattacharjee, V., Horn, K. H., Singh, S., Webb, C. L., Pisano, M. M. and Greene, R. M.** (2009). CBP/p300 and associated transcriptional co-activators exhibit distinct expression patterns during murine craniofacial and neural tube development. *Int. J. Dev. Biol.* **53**, 1097-1104.
- Blavier, L., Lazaryev, A., Groffen, J., Heisterkamp, N., DeClerck, Y. A. and Kaartinen, V.** (2001). TGF-beta3-induced palatogenesis requires matrix metalloproteinases. *Mol. Biol. Cell* **12**, 1457-1466.
- Boglev, Y., Wilanowski, T., Caddy, J., Parekh, V., Auden, A., Darido, C., Hislop, N. R., Cangkrama, M., Ting, S. B. and Jane, S. M.** (2011). The unique and cooperative roles of the Grainy head-like transcription factors in epidermal development reflect unexpected target gene specificity. *Dev. Biol.* **349**, 512-522.
- Brouns, M. R., De Castro, S. C., Terwindt-Rouvenhorst, E. A., Massa, V., Hekking, J. W., Hirst, C. S., Savery, D., Munts, C., Partridge, D., Lamers, W. et al.** (2011). Over-expression of *Grhl2* causes spina bifida in the Axial defects mutant mouse. *Hum. Mol. Genet.* **20**, 1536-1546.
- Bush, J. O. and Jiang, R.** (2012). Palatogenesis: morphogenetic and molecular mechanisms of secondary palate development. *Development* **139**, 231-243.
- Camenisch, T. D., Biesterfeldt, J., Brehm-Gibson, T., Bradley, J. and McDonald, J. A.** (2001). Regulation of cardiac cushion development by hyaluronan. *Exp. Clin. Cardiol.* **6**, 4-10.
- Camerer, E., Barker, A., Duong, D. N., Ganesan, R., Kataoka, H., Cornelissen, I., Darragh, M. R., Hussain, A., Zheng, Y. W., Srinivasan, Y. et al.** (2010). Local protease signaling contributes to neural tube closure in the mouse embryo. *Dev. Cell* **18**, 25-38.
- Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A. and Gruss, P.** (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94**, 727-737.
- Chen, B. and Hales, B. F.** (1995). Antisense oligonucleotide down-regulation of E-cadherin in the yolk sac and cranial neural tube malformations. *Biol. Reprod.* **53**, 1229-1238.
- Cohn, M. J.** (2011). Development of the external genitalia: conserved and divergent mechanisms of appendage patterning. *Dev. Dyn.* **240**, 1108-1115.
- Copp, A. J., Brook, F. A., Estibeiro, J. P., Shum, A. S. and Cockcroft, D. L.** (1990). The embryonic development of mammalian neural tube defects. *Prog. Neurobiol.* **35**, 363-403.
- Cuervo, R. and Covarrubias, L.** (2004). Death is the major fate of medial edge epithelial cells and the cause of basal lamina degradation during palatogenesis. *Development* **131**, 15-24.

- Cuervo, R., Valencia, C., Chandraratna, R. A. and Covarrubias, L. (2002). Programmed cell death is required for palate shelf fusion and is regulated by retinoic acid. *Dev. Biol.* **245**, 145-156.
- Dravis, C. and Henkemeyer, M. (2011). Ephrin-B reverse signaling controls septation events at the embryonic midline through separate tyrosine phosphorylation-independent signaling avenues. *Dev. Biol.* **355**, 138-151.
- Farbman, A. I. (1968). Electron microscope study of palate fusion in mouse embryos. *Dev. Biol.* **18**, 93-116.
- Ferretti, E., Li, B., Zewdu, R., Wells, V., Hebert, J. M., Karner, C., Anderson, M. J., Williams, T., Dixon, J., Dixon, M. J. et al. (2011). A conserved Pbx-Wnt-p63-Irf6 regulatory module controls face morphogenesis by promoting epithelial apoptosis. *Dev. Cell* **21**, 627-641.
- Fitchett, J. E. and Hay, E. D. (1989). Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Dev. Biol.* **131**, 455-474.
- Geelen, J. A. and Langman, J. (1979). Ultrastructural observations on closure of the neural tube in the mouse. *Anat. Embryol. (Berl.)* **156**, 73-88.
- Ghyselinck, N. B., Wendling, O., Messaddeq, N., Dierich, A., Lampron, C., Decimo, D., Viville, S., Chambon, P. and Mark, M. (1998). Contribution of retinoic acid receptor beta isoforms to the formation of the conotruncal septum of the embryonic heart. *Dev. Biol.* **198**, 303-318.
- Griffith, C. M. and Hay, E. D. (1992). Epithelial-mesenchymal transformation during palatal fusion: carboxyfluorescein traces cells at light and electron microscopic levels. *Development* **116**, 1087-1099.
- Guerrini, L., Costanzo, A. and Merlo, G. R. (2011). A symphony of regulations centered on p63 to control development of ectoderm-derived structures. *J. Biomed. Biotechnol.* **2011**, 864904.
- Gustavsson, P., Greene, N. D., Lad, D., Pauws, E., de Castro, S. C., Stanier, P. and Copp, A. J. (2007). Increased expression of Grainyhead-like-3 rescues spina bifida in a folate-resistant mouse model. *Hum. Mol. Genet.* **16**, 2640-2646.
- Harris, M. J. and Juriloff, D. M. (2010). An update to the list of mouse mutants with neural tube closure defects and advances toward a complete genetic perspective of neural tube closure. *Birth Defects Res. A Clin. Mol. Teratol.* **88**, 653-669.
- Henderson, D. J. and Copp, A. J. (1998). Versican expression is associated with chamber specification, septation, and valvulogenesis in the developing mouse heart. *Circ. Res.* **83**, 523-532.
- Hinrichsen, C. F. and Stevens, G. S. (1974). Epithelial morphology during closure of the secondary palate in the rat. *Arch. Oral Biol.* **19**, 969-980.
- Hinrichsen, K. (1985). The early development of morphology and patterns of the face in the human embryo. *Adv. Anat. Embryol. Cell Biol.* **98**, 1-79.
- Holder, A. M., Klaassens, M., Tibboel, D., de Klein, A., Lee, B. and Scott, D. A. (2007). Genetic factors in congenital diaphragmatic hernia. *Am. J. Hum. Genet.* **80**, 825-845.
- Holder, S. E., Vintiner, G. M., Farren, B., Malcolm, S. and Winter, R. M. (1992). Confirmation of an association between RFLPs at the transforming growth factor-alpha locus and non-syndromic cleft lip and palate. *J. Med. Genet.* **29**, 390-392.
- Holmberg, J., Clarke, D. L. and Frisen, J. (2000). Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. *Nature* **408**, 203-206.
- Iwata, J., Parada, C. and Chai, Y. (2011). The mechanism of TGF-beta signaling during palate development. *Oral Dis.* **17**, 733-744.
- Jankovics, F. and Brunner, D. (2006). Transiently reorganized microtubules are essential for zippering during dorsal closure in *Drosophila melanogaster*. *Dev. Cell* **11**, 375-385.
- Johnston, M. C. and Bronsky, P. T. (1995). Prenatal craniofacial development: new insights on normal and abnormal mechanisms. *Crit. Rev. Oral Biol. Med.* **6**, 25-79.
- Kitase, Y., Yamashiro, K., Fu, K., Richman, J. M. and Shuler, C. F. (2011). Spatiotemporal localization of periostin and its potential role in epithelial-mesenchymal transition during palatal fusion. *Cells Tissues Organs* **193**, 53-63.
- Knight, A. S., Schutte, B. C., Jiang, R. and Dixon, M. J. (2006). Developmental expression analysis of the mouse and chick orthologues of IRF6: the gene mutated in Van der Woude syndrome. *Dev. Dyn.* **235**, 1441-1447.
- Komatsu, K., Wakatsuki, S., Yamada, S., Yamamura, K., Miyazaki, J. and Sehara-Fujisawa, A. (2007). Meltrin beta expressed in cardiac neural crest cells is required for ventricular septum formation of the heart. *Dev. Biol.* **303**, 82-92.
- Koo, S. H., Cunningham, M. C., Arabshahi, B., Gruss, J. S. and Grant, J. H., 3rd (2001). The transforming growth factor-beta 3 knock-out mouse: an animal model for cleft palate. *Plast. Reconstr. Surg.* **108**, 938-951.
- Lee, J. M., Kim, J. Y., Cho, K. W., Lee, M. J., Cho, S. W., Kwak, S., Cai, J. and Jung, H. S. (2008). Wnt11/Fgfr1b cross-talk modulates the fate of cells in palate development. *Dev. Biol.* **314**, 341-350.
- Leonard, J. R., Klocke, B. J., D'Sa, C., Flavell, R. A. and Roth, K. A. (2002). Strain-dependent neurodevelopmental abnormalities in caspase-3-deficient mice. *J. Neuropathol. Exp. Neurol.* **61**, 673-677.
- Lidral, A. C., Murray, J. C., Buetow, K. H., Basart, A. M., Scheerer, H., Shiang, R., Naval, A., Layda, E., Magee, K. and Magee, W. (1997). Studies of the candidate genes TGFB2, MSX1, TGFA, and TGFB3 in the etiology of cleft lip and palate in the Philippines. *Cleft Palate Craniofac. J.* **34**, 1-6.
- Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Dierich, A., Gorry, P., Gansmuller, A. and Chambon, P. (1994). Function of the retinoic acid receptors (RARs) during development (I). Craniofacial and skeletal abnormalities in RAR double mutants. *Development* **120**, 2723-2748.
- Martinez-Alvarez, C., Tudela, C., Perez-Miguelsanz, J., O'Kane, S., Puerta, J. and Ferguson, M. W. (2000). Medial edge epithelial cell fate during palatal fusion. *Dev. Biol.* **220**, 343-357.
- Massa, V., Savery, D., Ybot-Gonzalez, P., Ferraro, E., Rongvaux, A., Cecconi, F., Flavell, R., Greene, N. D. and Copp, A. J. (2009). Apoptosis is not required for mammalian neural tube closure. *Proc. Natl. Acad. Sci. USA* **106**, 8233-8238.
- Matteson, P. G., Desai, J., Korstanje, R., Lazar, G., Borsuk, T. E., Rollins, J., Kadambi, S., Joseph, J., Rahman, T., Wink, J. et al. (2008). The orphan G protein-coupled receptor, Gpr161, encodes the vacuolated lens locus and controls neurulation and lens development. *Proc. Natl. Acad. Sci. USA* **105**, 2088-2093.
- McLaughlin, M. E., Kruger, G. M., Slocum, K. L., Crowley, D., Michaud, N. A., Huang, J., Magendanz, M. and Jacks, T. (2007). The Nf2 tumor suppressor regulates cell-cell adhesion during tissue fusion. *Proc. Natl. Acad. Sci. USA* **104**, 3261-3266.
- Millicovsky, G. and Johnston, M. C. (1981). Active role of embryonic facial epithelium: new evidence of cellular events in morphogenesis. *J. Embryol. Exp. Morphol.* **63**, 53-66.
- Mo, F. E. and Lau, L. F. (2006). The matricellular protein CCN1 is essential for cardiac development. *Circ. Res.* **99**, 961-969.
- Mori, C., Nakamura, N., Okamoto, Y., Osawa, M. and Shiota, K. (1994). Cytochemical identification of programmed cell death in the fusing fetal mouse palate by specific labelling of DNA fragmentation. *Anat. Embryol. (Berl.)* **190**, 21-28.
- Nottoli, T., Hagopian-Donaldson, S., Zhang, J., Perkins, A. and Williams, T. (1998). AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. *Proc. Natl. Acad. Sci. USA* **95**, 13714-13719.
- Orioli, D., Henkemeyer, M., Lemke, G., Klein, R. and Pawson, T. (1996). Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. *EMBO J.* **15**, 6035-6049.
- Pexieder, T. (1975). Cell death in the morphogenesis and teratogenesis of the heart. *Adv. Anat. Embryol. Cell Biol.* **51**, 3-99.
- Pexieder, T. (1978). Development of the outflow tract of the embryonic heart. *Birth Defects Orig. Artic. Ser.* **14**, 29-68.
- Poelmann, R. E., Mikawa, T. and Gittenberger-de Groot, A. C. (1998). Neural crest cells in outflow tract septation of the embryonic chicken heart: differentiation and apoptosis. *Dev. Dyn.* **212**, 373-384.
- Proetzl, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W. and Doetschman, T. (1995). Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat. Genet.* **11**, 409-414.
- Pyrgaki, C., Trainor, P., Hadjantonakis, A. K. and Niswander, L. (2010). Dynamic imaging of mammalian neural tube closure. *Dev. Biol.* **344**, 941-947.
- Pyrgaki, C., Liu, A. and Niswander, L. (2011). Grainyhead-like 2 regulates neural tube closure and adhesion molecule expression during neural fold fusion. *Dev. Biol.* **353**, 38-49.
- Qu, S., Tucker, S. C., Zhao, Q., deCrombrughe, B. and Wisdom, R. (1999). Physical and genetic interactions between Alx4 and Cart1. *Development* **126**, 359-369.
- Richardson, R. J., Dixon, J., Jiang, R. and Dixon, M. J. (2009). Integration of IRF6 and Jagged2 signalling is essential for controlling palatal adhesion and tissue competence. *Hum. Mol. Genet.* **18**, 2632-2642.
- Rifat, Y., Parekh, V., Wilanowski, T., Hislop, N. R., Auden, A., Ting, S. B., Cunningham, J. M. and Jane, S. M. (2010). Regional neural tube closure defined by the Grainy head-like transcription factors. *Dev. Biol.* **345**, 237-245.
- Risley, M., Garrod, D., Henkemeyer, M. and McLean, W. (2009). EphB2 and EphB3 forward signalling are required for palate development. *Mech. Dev.* **126**, 230-239.
- San Miguel, S., Serrano, M. J., Sachar, A., Henkemeyer, M., Svoboda, K. K. and Benson, M. D. (2011). Ephrin reverse signaling controls palate fusion via a PI3 kinase-dependent mechanism. *Dev. Dyn.* **240**, 357-364.
- Schorle, H., Meier, P., Buchert, M., Jaenisch, R. and Mitchell, P. J. (1996). Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* **381**, 235-238.
- Senders, C. W., Peterson, E. C., Hendrickx, A. G. and Cukierski, M. A. (2003). Development of the upper lip. *Arch. Facial Plast. Surg.* **5**, 16-25.
- Shaut, C. A., Saneyoshi, C., Morgan, E. A., Knosp, W. M., Sexton, D. R. and Stadler, H. S. (2007). HOXA13 directly regulates EphA6 and EphA7 expression in the genital tubercle vascular endothelium. *Dev. Dyn.* **236**, 951-960.
- Stephen, L. J., Fawkes, A. L., Verhoeve, A., Lemke, G. and Brown, A. (2007). A critical role for the EphA3 receptor tyrosine kinase in heart development. *Dev. Biol.* **302**, 66-79.
- Sun, D., Baur, S. and Hay, E. D. (2000). Epithelial-mesenchymal transformation is the mechanism for fusion of the craniofacial primordia involved in morphogenesis of the chicken lip. *Dev. Biol.* **228**, 337-349.

- Takahara, S., Takigawa, T. and Shiota, K.** (2004). Programmed cell death is not a necessary prerequisite for fusion of the fetal mouse palate. *Int. J. Dev. Biol.* **48**, 39-46.
- Takeuchi, J. K., Ohgi, M., Koshiba-Takeuchi, K., Shiratori, H., Sakaki, I., Ogura, K., Saijoh, Y. and Ogura, T.** (2003). Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis. *Development* **130**, 5953-5964.
- Thompson, R. P., Sumida, H., Abercrombie, V., Satow, Y., Fitzharris, T. P. and Okamoto, N.** (1985). Morphogenesis of human cardiac outflow. *Anat. Rec.* **213**, 578-586.
- Ting, S. B., Wilanowski, T., Auden, A., Hall, M., Voss, A. K., Thomas, T., Parekh, V., Cunningham, J. M. and Jane, S. M.** (2003). Inositol- and folate-resistant neural tube defects in mice lacking the epithelial-specific factor Grhl-3. *Nat. Med.* **9**, 1513-1519.
- Vaziri Sani, F., Hallberg, K., Harfe, B. D., McMahon, A. P., Linde, A. and Gritli-Linde, A.** (2005). Fate-mapping of the epithelial seam during palatal fusion rules out epithelial-mesenchymal transformation. *Dev. Biol.* **285**, 490-495.
- Vuillemin, M. and Pexieder, T.** (1989). Normal stages of cardiac organogenesis in the mouse: II. Development of the internal relief of the heart. *Am. J. Anat.* **184**, 114-128.
- Waldo, K., Miyagawa-Tomita, S., Kumiski, D. and Kirby, M. L.** (1998). Cardiac neural crest cells provide new insight into septation of the cardiac outflow tract: aortic sac to ventricular septal closure. *Dev. Biol.* **196**, 129-144.
- Wansleben, C., Feitsma, H., Montcouquiol, M., Kroon, C., Cuppen, E. and Meijlink, F.** (2010). Planar cell polarity defects and defective Vangl2 trafficking in mutants for the COPII gene Sec24b. *Development* **137**, 1067-1073.
- Webb, S., Brown, N. A. and Anderson, R. H.** (1998). Formation of the atrioventricular septal structures in the normal mouse. *Circ. Res.* **82**, 645-656.
- Webb, S., Qayyum, S. R., Anderson, R. H., Lamers, W. H. and Richardson, M. K.** (2003). Septation and separation within the outflow tract of the developing heart. *J. Anat.* **202**, 327-342.
- Weil, M., Jacobson, M. D. and Raff, M. C.** (1997). Is programmed cell death required for neural tube closure? *Curr. Biol.* **7**, 281-284.
- Wenink, A. C. and Zavallos, J. C.** (1988). Developmental aspects of atrioventricular septal defects. *Int. J. Cardiol.* **18**, 65-78.
- Werth, M., Walentin, K., Aue, A., Schonheit, J., Wuebken, A., Pode-Shakked, N., Vilianovitch, L., Erdmann, B., Dekel, B., Bader, M. et al.** (2010). The transcription factor grainyhead-like 2 regulates the molecular composition of the epithelial apical junctional complex. *Development* **137**, 3835-3845.
- Yamaguchi, Y., Shinotsuka, N., Nonomura, K., Takemoto, K., Kuida, K., Yosida, H. and Miura, M.** (2011). Live imaging of apoptosis in a novel transgenic mouse highlights its role in neural tube closure. *J. Cell Biol.* **195**, 1047-1060.
- Ybot-Gonzalez, P., Savery, D., Gerrelli, D., Signore, M., Mitchell, C. E., Faux, C. H., Greene, N. D. and Copp, A. J.** (2007). Convergent extension, planar-cell-polarity signalling and initiation of mouse neural tube closure. *Development* **134**, 789-799.
- Yu, H., Smallwood, P. M., Wang, Y., Vidaltamayo, R., Reed, R. and Nathans, J.** (2010). Frizzled 1 and frizzled 2 genes function in palate, ventricular septum and neural tube closure: general implications for tissue fusion processes. *Development* **137**, 3707-3717.
- Zeng, H., Hoover, A. N. and Liu, A.** (2010). PCP effector gene Inturned is an important regulator of cilia formation and embryonic development in mammals. *Dev. Biol.* **339**, 418-428.
- Zhang, J., Hagopian-Donaldson, S., Serbedzija, G., Elsemore, J., Plehn-Dujowich, D., McMahon, A. P., Flavell, R. A. and Williams, T.** (1996). Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature* **381**, 238-241.
- Zhao, Z. and Rivkees, S. A.** (2000). Programmed cell death in the developing heart: regulation by BMP4 and FGF2. *Dev. Dyn.* **217**, 388-400.