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REVIEW

Craniofacial transitions: the role of EMT and MET during head development

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ABSTRACT

Within the developing head, tissues undergo cell-fate transitions to shape the forming structures. This starts with the neural crest, which undergoes epithelial-to-mesenchymal transition (EMT) to form, amongst other tissues, many of the skeletal tissues of the head. In the eye and ear, these neural crest cells then transform back into an epithelium, via mesenchymal-to-epithelial transition (MET), highlighting the flexibility of this population. Elsewhere in the head, the epithelium loses its integrity and transforms into mesenchyme. Here, we review these craniofacial transitions, looking at why they happen, the factors that trigger them, and the cell and molecular changes they involve. We also discuss the consequences of aberrant EMT and MET in the head.

KEY WORDS: Mesenchyme, Epithelium, Endothelium, Neural crest, Cell fate

Introduction

Cellular plasticity is of vital importance in development, disease and repair. One particular example of this plasticity, epithelial-to-mesenchymal transition (EMT), and its reverse, mesenchymal-to-epithelial transition (MET), is key to many stages of development and disease (Yang et al., 2020). Epithelial cells form single or multilayer structures with apical-basal cell polarity. They adhere to and communicate with each other through specialised junctions, which are a primary feature of epithelial cell identity (Blanpain et al., 2007; Knust, 2002). Epithelial cells can form permeable or impermeable membranes in this fashion and, therefore, define the borders of tissues and organs. Conversely, mesenchymal cells lack these defined cell-to-cell junctions and are capable of migration using front-rear polarity (Hay, 2005). They also have altered cell cytoskeletons in order to aid their motility (Hay, 2005). However, these cell states – epithelial or mesenchymal – are not always fixed, and cells can switch between them via EMT and MET. Cells can also undergo partial EMT or MET, thereby displaying both epithelial and mesenchymal characteristics and residing in an intermediary cell state (Sha et al., 2019). It is important to recognise that cells can exhibit this form of plasticity when prescribing a cell type to a tissue to avoid mistakenly identifying EMT or MET when it may only be a transient state (Yang et al., 2020).

The tissues of the head form extremely complex structures derived from multiple origins. These structures show great diversity across vertebrates and form cranial structures that are important for life, such as the sensory organs, feeding apparatus and support for the brain. To create many of these intricate cranial organs, both EMT and MET are essential. Here, we discuss how EMT and MET play a crucial role in creating and shaping the vertebrate head. We begin by providing an overview of the molecular basis of EMT and MET, and highlight the importance of EMT in the neural crest – a tissue that gives rise to a number of vertebrate craniofacial structures within the head, including the eye, ear, palate and teeth. We focus on findings in mammals, predominantly mouse models, which have the advantage of the ability to lineage trace, although we also briefly cover other animal models such as the mini pig, which has been used in tooth research. Where applicable, we also highlight how perturbations in EMT/MET have been linked to developmental disorders or disease.

The molecular basis of epithelial-mesenchymal transitions

Cells undergoing EMT must redirect their gene expression profile in several ways (Fig. 1). A key characteristic of epithelial cells is their cell-cell junctions, particularly tight junctions, adherens junctions, desmosomes and gap junctions (Pinheiro and Bellaïche, 2018), which must be removed to allow an epithelial cell to adopt a mesenchymal fate. An important change in cells undergoing EMT is the downregulation of E-cadherin, which contributes to the breakdown of cadherin junctions (Gheldof and Berx, 2013). This is accompanied by a switch to the expression of N-cadherins, which are markers of a mesenchymal phenotype and are used for motility, forming connections with the cytoskeleton (Gheldof and Berx, 2013).

Another defining characteristic of epithelial cells is their apical-basal polarity. This is mediated through polarity protein complexes, namely scribble, crumbs and partitioning defective (Par) complexes (Wen and Zhang, 2018). These protein complexes localise to specific areas of the plasma membrane, thereby providing asymmetry and polarity within the cell (Ngok et al., 2014). Polarity complexes help to stabilise cell-cell adhesions in epithelial cells, and an early sign of EMT is the destabilisation of polarity complexes, leading to the breakdown of cell-cell adhesions (Ozdamar et al., 2005).

Genes governing the cytoskeleton must also change in order to allow motility in mesenchymal cells. Cytokeratin is downregulated and, in turn, vimentin is upregulated, allowing differences in the interactions at the cell surface to promote motility (Liu et al., 2015). Mesenchymal cells interact with and can degrade the extracellular matrix (ECM) in order to promote their own motility. This is mediated through the up- and downregulation of specific integrins (Docheva et al., 2007). Mesenchymal cells can also secrete matrix metalloproteases (MMPs) to remodel the ECM and thereby promote their motility (Gonzalez and Medici, 2014; McGrail et al., 2015).

EMT initiation and control is driven by various transcription factors. The most widely studied of these are Snail, TWIST and ZEB, although other transcription factors are also capable of driving EMT (Stemmler et al., 2019). The transcription factor controlling a specific EMT is often tissue- or context-dependent, although many of the same signalling pathways are activated by these transcription factors, and they are also capable of regulating one another,

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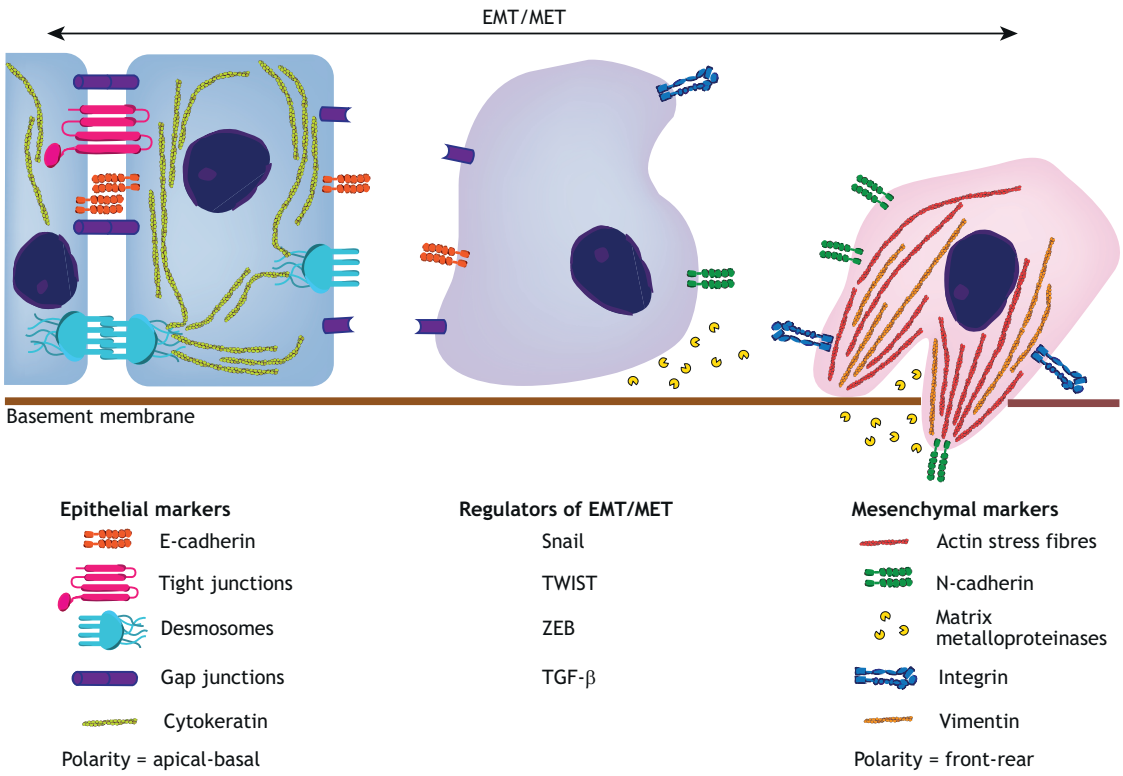

Fig. 1. A summary of the molecular changes occurring during EMT/MET. Epithelial cells (left) are characterised by their cell-cell junctions and apical-basal cell polarity. By contrast, mesenchymal cells (right) are characterised by their motility, polarity (which is front-rear in their direction of movement) and their ability to remodel the ECM in line with their motility. EMT, and its reverse process MET, is regulated by a number of transcription factors. Factors that promote EMT trigger the upregulation of mesenchymal-specific genes, control mesenchymal-specific cell junctions and increase the expression of molecules enabling cell motility. This establishes front-to-rear polarity in mesenchymal cells and is a hallmark of mesenchymal cell identity.

demonstrating a high level of interdependence (Dave et al., 2011; Stemmler et al., 2019).

TGF-β family proteins are also important initiators of EMT, both in development and in wound healing (Schmierer and Hill, 2007). They bind to cell membranes and activate intracellular signalling networks, of which the SMAD family of proteins is a primary component (Schmierer and Hill, 2007). TGF-β can also be activated from the surrounding ECM, where it resides in a latent form. This activation is triggered by MMPs as they remodel the ECM (Lamouille et al., 2014). SMAD proteins also activate Snail transcription factors (Snail and Slug, also known as Snail1 and Snail2, respectively), which are key regulators of EMT and can also trigger EMT independently of their activation by SMADs (Cano et al., 2000). They do this by suppressing epithelial genes through zinc-finger domains and through activating MMPs (Cano et al., 2000). Snail has recently been shown to repress expression of the transcription factor Prrx1, and these two factors form a mutual regulatory network and are expressed in complementary patterns during development (Fazilaty et al., 2019). This provides patterning information to migrating mesenchymal cells, and has been demonstrated to help maintain cell fate within the craniofacial mesenchyme (Lu et al., 1999). Several pathways are known to inhibit Snail proteins, including the WNT signalling pathway, which acts by inhibiting Snail phosphorylation (Zhou et al., 2004). In addition, Notch expression can inhibit Snail signalling by inhibiting the interaction between Snail and GSK3β, thereby destabilising the Snail protein (Zhou et al., 2004).

Basic helix-loop-helix (bHLH) proteins such as TWIST1 and 2, E12 and E47 are also powerful regulators of EMT (Lamouille et al., 2014). Similar to Snail proteins, TWIST proteins repress E-cadherins and promote the expression of N-cadherins (Bai et al., 2017). TWIST is therefore an important activator of differentiation in multiple tissues, with activation in some instances linked to mechanical stress (Zhu et al., 2016). Both TWIST and Snail can activate ZEB transcription factors (Peinado et al., 2007), which in turn can act to either supress or activate EMT, depending on the molecules they are co-factored with (Peinado et al., 2007). The action of EMT/MET-associated transcription factors is therefore often context- or organ-dependent and can be regulated by different physical and genetic cues during development. Moreover, because such EMT/MET-associated factors are often expressed only transiently, tracking when and where EMT/MET occurs remains a challenge (see Box 1).

The neural crest: building blocks of the vertebrate head

The neural crest is a population of cells that is unique to vertebrate development and is highly conserved between vertebrate species (Kuratani, 2018). During development, the neural crest becomes regionalised along the anterior-posterior axis into subpopulations with distinct differentiation potential: cranial, vagal, trunk and sacral. The majority of the vertebrate head skeleton (connective and skeletal tissues) is derived from cranial neural crest (Couly et al., 1993). Indeed, reporter mice have confirmed that the cranial neural crest contributes to the mandible, parts of the ear, teeth, neurons, glia, cartilage and other connective tissues in the head (Chai et al., 2000; Morriss-Kay, 2001). These tissues form important structures that allowed vertebrates to occupy diverse ecological niches and triggered the evolution of a huge diversity of craniofacial features within the vertebrate phylum (Green et al., 2015; Schneider, 2018; Wu et al.,

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Box 1. Tracking EMT and MET experimentally

Once a cell has undergone an EMT or MET, evidence of its former cell state is often completely removed. Therefore, tracking cell-fate changes has been fraught with difficulty, relying on the identification of a transition stage in which markers of both epithelial and mesenchymal identity are evident (Buchtová et al., 2012). In early studies, the grafting of cells between animals of closely related species before EMT/MET was used to probe the fate of different cell populations (Couly et al., 1993; Horstadius, 1950). As techniques to trace cell fate improved, researchers turned to the use of GFP-labelled reporters for within-species grafts, for example in mouse, chick, *Xenopus*, zebrafish and axolotl embryos (Amsterdam et al., 1995; Mozdziak and Petitte, 2004; Sobkow et al., 2006; Zernicka-Goetz et al., 1996, 1997). Modern-day advances in lineage tracing, together with the generation of transgenic mice, such as those harbouring inducible recombinases and multi-colour reporter constructs, allow for much more sophisticated techniques for tracking and understanding cell fate (Kretzschmar and Watt, 2012). These techniques have been applied successfully to study epithelial and mesenchymal populations and, hence, are useful for tracking EMT/MET. For example, *Wnt1cre* is routinely used to track neural crest cell fate, whereas keratins 5 and 14 (K5 and K14) are widely used to track epithelial cell fate (Hari et al., 2012; Van Keymeulen and Blanpain, 2012). The complexity and sophistication of such reporter lines is also increasing. In addition, single cell resolution is now achievable when probing gene expression in lineage-traced cells (Kester and van Oudenaarden, 2018), and CRISPR/Cas9 gene editing can be used to individually label cells without the need for fluorescent reporters (Spanjaard et al., 2018).

2017). The cells of the cranial neural crest migrate in two waves, and the timing of this migration correlates with the fate of these cells (Graham et al., 2004). Early migratory cells populate the pharyngeal arches and facial prominences, whereas later migrating cranial neural crest cells remain closer to the central nervous system and go on to form the neurons and glia of the head (Baker et al., 1997).

The neural crest originates from the ectoderm, forming at the border between the neural plate and the non-neural plate ectoderm (Prasad et al., 2019). The cells that are to become neural crest cells are determined by extrinsic signalling factors such as Wnt, FGF, BMP and Notch, which are expressed by surrounding tissues (Prasad et al., 2019). Cells receiving these signalling factors are then induced to express a set of neural plate border specifiers, such as Dlx3, Dlx5, Pax3, Pax7 and Zic (Plouhinec et al., 2014). These cells then start to express neural crest-specific genes (Sauka-Spengler and Bronner-Fraser, 2006), a combination of which can be used to identify these cells. Examples of general neural crest markers include Snail2, Sox10, FoxD3 and Sox9 (Morrison et al., 2017), while Sox8 and Ets1 specify cranial neural crest cells early in development and are maintained throughout the migration of the cell from the neural tube (Simoes-Costa and Bronner, 2016).

As they become specified, neural crest cells delaminate from the neural plate and undergo EMT, enabling them to migrate and contribute to a diverse range of tissues throughout the embryo (Sauka-Spengler and Bronner-Fraser, 2008). Tightly controlled Wnt and BMP signalling is essential for migration of the cranial neural crest (Burstyn-Cohen et al., 2004). Downstream of these pathways, Snail, TWIST, Sox10 and FoxD3 regulate EMT, which allows this migration through control of cadherin and MMP expression, and reorganisation of the cytoskeleton (Clay and Halloran, 2010; Dady et al., 2012). Specifically, the cleavage of E-cadherins and the switch to N-cadherins may be modulated by MMP14 expression (Garmon et al., 2018).

In addition to EMT of the neural crest, which allows its migration into the head, several organs within the head involve an EMT and MET as they form. These include the corneal endothelium within the eye, the middle ear, the palate and the teeth.

The corneal endothelium

The cornea forms the structural barrier and an important refractive component of the eye. It is made of several layers, primarily the corneal epithelium, the central stroma and the corneal endothelium (DelMonte and Kim, 2011). The corneal endothelium is a monolayer that, in adults, maintains the appropriate levels of dehydration within the corneal stroma that are required for tissue clarity (Eghrari et al., 2015). Several cell-state transitions are involved in the corneal endothelium in both development and disease (Cvekl and Tamm, 2004). As the eye develops, mesenchymal-to-endothelial transition (MEndoT, a process related to MET/EMT; see Box 2) takes place to generate a functional endothelium (Fig. 2A). During injury and subsequent wound healing, the reverse process, endothelial-to-mesenchymal transition (EndoMT; see Box 2), occurs (Lee et al., 2019; Walshe et al., 2018). In developmental disorders of the corneal endothelium, aberrant expression of genes controlling these cell-state transitions is often culpable (Frausto et al., 2016; Zakharevich et al., 2017).

The layers of the cornea develop secondary to the separation of the lens vesicle from the surface ectoderm. The mesenchymal cells that form the corneal endothelium are derived from the neural crest (Gage et al., 2005). After the separation of the lens vesicle, these mesenchymal cells migrate into the space created between the two tissues (Cvekl and Tamm, 2004). As they proliferate, they form discrete sheets of cells. The layer of cells closest to the lens flattens and extends to form junctions with neighbouring cells, forming the endothelial monolayer of the cornea, undergoing MEndoT in the process (Cvekl and Tamm, 2004). The remaining layers of mesenchymal cells go on to form the corneal stroma (Gage et al., 2005).

The transition to an endothelial cell type involves the expression of tight junctions, which are essential for maintenance of the endothelial cell barrier (Eghrari et al., 2015). However, although cells of the endothelium exhibit epithelial organisation in terms of their cell-cell connections, they do not upregulate all the markers key to epithelial cell function, and therefore appear to be in a transitionary state between an epithelial and mesenchymal cell type (Frausto et al., 2016). For example, corneal endothelial cells express high levels of N-cadherins, which are classical markers of

Box 2. EndoMT and MEndoT

Endothelial-to-mesenchymal transition (EndoMT) and its reverse, mesenchymal-to-endothelial transition (MEndoT), are cell-state transitions that are involved in specific aspects of development, disease and repair. MEndoT is involved in the condensation of neural crest cells to endothelial cell types, an example of which includes the corneal endothelium (Gage et al., 2005). EndoMT and MEndoT are also heavily involved in heart development (Plein et al., 2015; von Gise and Pu, 2012), and MEndoT may play a role in remodelling of the heart after injury. Indeed, a subset of fibroblasts has been found to adopt an endothelial cell type after injury in the heart to aid restoration of vascular density (Ubil et al., 2014). Some of the signalling pathways involved in EndoMT and MEndoT are the same as those found in EMT and MET. For example, the gene *p53* (*Trp53*) can regulate EMT and is also widely expressed in fibroblast derived-endothelial cells in the heart (Ubil et al., 2014). EndoMT has also been suggested as a driving factor behind more general fibrotic disorders such as lung and renal fibrosis, and multiple signalling pathways involved in EMT, such as the TGF-β, Notch and Wnt pathways, are also postulated to drive this transition (Piera-Velazquez et al., 2016).

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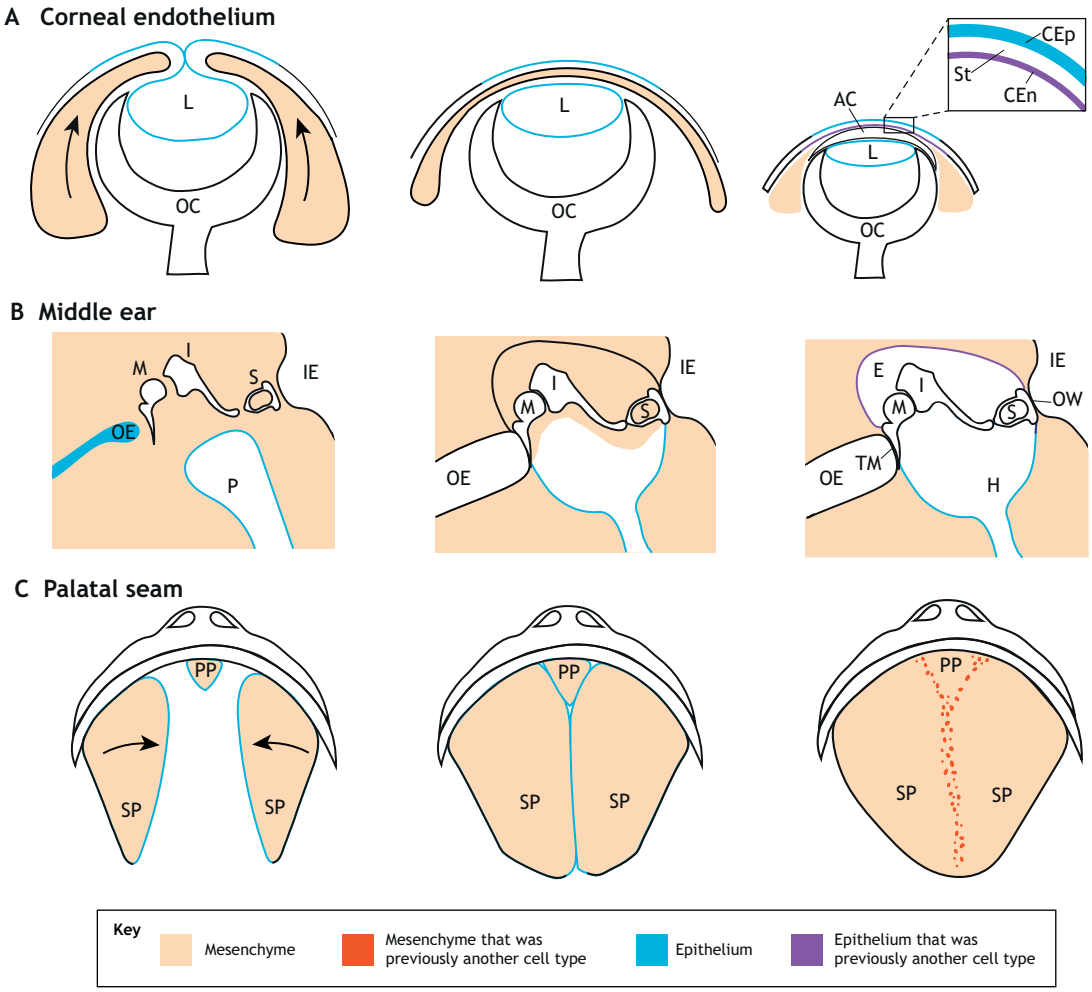

Fig. 2. EMT and MET in the vertebrate head. (A-C) The development of many structures within the vertebrate head involves either EMT or MET. These structures are often extremely specialised, and many are unique to vertebrates. Schematics show development of the corneal endothelium (A), the middle ear (B) and the palatal seam (C). AC, anterior chamber; CEn, corneal endothelium; CEp, epithelium; E, epitympanic region; H, hypotympanic region; I, incus; IE, inner ear; L, lens; M, malleus; OC, optic cup; OE, outer ear; OW, oval window; P, pharyngeal pouch; PP, primary palate; S, stapes; SP, secondary palate; St, stroma; TM, tympanic membrane.

mesenchymal cells (Reneker et al., 2000). Interestingly, N-cadherin is essential for function of the endothelium, with N-cadherin-null corneal endothelial cells exhibiting extensive cytoskeletal changes and an inability to form proper cell junctions (Vassilev et al., 2012).

Cell-state transitions also play an important role in regenerative processes within the cornea. Mature endothelial cells of the cornea are normally under cell cycle arrest (Joyce et al., 2002). If cells are lost, however, the remaining cells either increase in size to compensate (Ljubimov and Saghizadeh, 2015) or undergo cell migration (Walshe et al., 2018). Indeed, migration appears to be key to wound healing in the corneal endothelium and is mediated through FGF2-directed EndoMT. Accordingly, EndoMT markers such as Snail, Zeb1 and vimentin are not expressed when FGF2 is knocked out in corneal endothelial cells, and the capacity of the tissue to heal is reduced (Lee et al., 2019).

Cell-state transitions, and the genes that control them, are also implicated in diseases of the cornea. For example, posterior polymorphous corneal dystrophy is a heritable corneal disorder characterised by *ZEB1* insufficiency (Aldave et al., 2013). This causes the corneal endothelium to exhibit more epithelial-like characteristics, particularly in terms of its cell junctions. For example, the expression of desmosomal junctions and the expression of E-cadherins and keratins is increased (Frausto et al., 2019). This phenotype results in progressive corneal oedema, caused by an influx of fluids through the compromised endothelium, and reduced visual acuity (Frausto et al., 2016). Modelling the disease in cell culture by knocking down *ZEB1* revealed an increase in UV-mediated apoptosis and an increased epithelial cell-like morphology. This demonstrates that, although the epithelium-like characteristics of the corneal endothelium are vital for its function, 'pushing' cell phenotype too far from a mesenchymal phenotype results in improper cell function (Zakharevich et al., 2017).

Fuchs endothelial corneal dystrophy (FECD) is another heritable disorder of the corneal endothelium (Matthaei et al., 2019). It is one of the most common disorders of the endothelium and is characterised by age-related loss of endothelial cells and a build-up of ECM, akin to scar formation (Jurkunas, 2018). The formation of these ECM deposits is mediated by EMT, and classical mesenchymal markers of the corneal endothelium such as Snail1, ZEB1 and fibronectin are upregulated in FECD (Katikireddy et al., 2018). Corneal endothelium cells therefore appear to be at a balance point between mesenchymal and epithelial fates and can be driven in either direction due to changes in signalling during repair or disease.

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Understanding these cell-state transitions in the cornea has important implications for regenerative medicine. For example, therapies for corneal endothelial regeneration focus on growing an endothelial layer *in vitro*, but a key obstacle is the tendency for these cells to undergo EMT and thereby lose their function (Ho et al., 2015). The modulation of MMPs is integral to this process in the corneal endothelium, so inhibition of MMP activity may be an important tool in retaining corneal endothelial function (Ho et al., 2015). TGF-β also appears to have multiple roles in endothelial cell homeostasis. It may influence cells to migrate during injury (Sumioka et al., 2008) and can induce proliferating endothelial cells to transition back to a mesenchymal phenotype (Beaulieu Leclerc et al., 2018). TGF-β2 is also required to inhibit cell proliferation within the endothelium and plays a role in inhibiting contact inhibition between cells (Joyce et al., 2002).

The middle ear

The mammalian middle ear contains three ossicles that reside within an air-filled cavity (Fuchs and Tucker, 2015). It has been proposed that the different parts of the cavity are lined by mucosa of two different origins – endoderm and neural crest (Thompson and Tucker, 2013) – with MET of the neural crest being required to convert the neural crest-derived cells into a mucosal lining (Fig. 2B).

The formation of an air-filled cavity lined continuously with epithelium is a developmentally complex process; a continuous expansion of an air-filled cavity would be difficult because of the intervening ossicles, muscles and nerves of the middle ear (Proctor, 1964). Therefore, a neural crest-derived epithelium represents a solution for providing an epithelium deep within the head. In line with this, it has been shown that, from the initiation of the structures of the middle ear [at around embryonic day (E) 12.5 in the mouse] to early postnatal development, the area that will go on to form the air-filled cavity of the middle ear is filled with mesenchymal cells derived from the neural crest (Fuchs and Tucker, 2015). Several hypotheses for the fate of these mesenchymal cells during cavitation have been proposed, one of which is apoptosis; however, studies show only a small fraction of cells undergo this fate, and instead a redistribution of mesenchymal tissue, as the bony bulla that houses the middle ear grows, has been proposed (Piza et al., 1998; Roberts and Miller, 1998).

More recent lineage-tracing studies show that neural crest-derived cells (Wnt1cre-positive) line the attic region of the middle ear in the adult (Thompson and Tucker, 2013). For this to happen, these neural crest cells must undergo MET during cavitation, which begins in early postnatal development in the mouse and is largely complete by postnatal day (P) 14 (Richter et al., 2010). Accordingly, epithelial markers such as E-cadherin and keratin 14 (K14) are expressed in neural crest-derived cells as cavitation is completed, between P14 and P19 (Thompson and Tucker, 2013). The signals that trigger this MET are unknown but could involve mechanical as well as signalling cues. When early postnatal rat middle ear mesenchyme is cultured *in vitro*, mesenchymal cells from the attic change their stellate shape and adopt an epithelial morphology after 8 days (Foley et al., 1965), suggesting that the process of MET occurs spontaneously in this context. Interestingly, the epithelioid cells revert to a stellate form when sub-cultured, suggesting that the MET that occurs in culture does not induce a permanent state and highlighting the potential flexibility of these cells (Foley et al., 1965).

Endoderm-derived cells contribute to the ventral mucosal lining of the middle ear and are continuous with the Eustachian tube and the pharynx (Thompson and Tucker, 2013). How the endodermal and neural crest-lined parts of the middle ear cavity link up during development, and at what stage, is not understood. An early break in the endoderm (at around E15.5 in the mouse) has been proposed (Thompson and Tucker, 2013), with the neural crest invading the space of the first pharyngeal pouch before retracting. However, further investigation is required to support this theory and to determine whether the break occurs at this time point or later, when the attic starts to form. A thin epithelium has been shown to line the retracting mesenchyme during the cavitation process, but the origin of these cells has not yet been assessed, and it is not known whether they contribute to the final neural crest-derived epithelium (Del-Pozo et al., 2019). Studies of human foetal samples suggest an endodermal only-derived lining for the whole cavity, but these analyses were performed before cavitation of the attic and before the formation of an attic mucosa lining (van Waegeningh et al., 2019). Notably, the aerated spaces in the attic and mastoid of the human middle ear are not completed until 4 years of age, indicating a highly complex system with extended development in humans (Miyanaga and Morimitsu, 1997).

The importance of appropriate MET in the middle ear is highlighted by cavitation defects, which are linked to middle ear disease and hearing loss (Del-Pozo et al., 2019; Miyanaga and Morimitsu, 1997). In some patients, mesenchyme can persist in the middle ear until adulthood. In patients with persistent mesenchyme, over 90% showed evidence of otitis media (middle ear infection) (Jaisinghani et al., 1999). This suggests a relationship between the two, although whether the retention of mesenchyme is a cause of otitis media or whether infection and inflammation cause the presence of these mesenchymal cells requires further investigation. In the *FBX011* mouse model, chronic otitis media is initiated by a bulla cavitation defect, highlighting defects in cavitation as the driver for otitis media in this case (Del-Pozo et al., 2019).

A similar link has been shown in *Cdh11* knockout mice (Kiyama et al., 2018). Cdh11 is expressed in neural crest-derived cells and is involved in cell fate and morphogenesis (Kimura et al., 1995), and so may be involved in MET, or aberrant MET, in the middle ear. *Cdh11* was previously found to be involved in EMT in the lung during remodelling in response to disease, and in EMT in several cancers (Chen et al., 2019; Wang et al., 2020). *Cdh11* knockout mice present with moderate hearing loss and a middle ear cavitation defect; the air-filled cavity of the middle ear in mutants is significantly reduced or completely lost, and mesenchymal cells in the middle ear persist into adulthood. These mice also often present with otitis media, pointing to a key role for MET in susceptibility to otitis media (Kiyama et al., 2018).

In other mouse models of otitis media, such as *Eya4*−/− mutant mice, there is also retention of mesenchymal cells in the middle ear (Depreux et al., 2008). In cases where the mesenchyme is retained, normal MET may not occur, thereby hindering the creation of an epithelium and making the middle ear susceptible to damage. The links between otitis media, failure in cavitation and MET are therefore a potential area for further exploration. Interestingly, aberrant EMT as well as MET may also cause disease in the middle ear. For example, cholesteatoma, a benign middle ear cyst, is associated with increased EMT mediated by p63, leading to increased epithelial cell division via the acquisition of a semi-mesenchymal cell state (Takahashi et al., 2019).

The palatal seam

The palate forms from two shelves that must fuse together in the midline to create a seamless tissue covering the roof of the mouth (Fig. 2C). Correct fusion of the palatal seam is essential for the ability to both feed and communicate. The mechanisms behind this

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fusion have long been debated and involve removal of the epithelial seam by apoptosis, cell migration or EMT, or a combination of the three (Nawshad, 2008).

During normal development, the mammalian upper jaw arises from six main prominences: two central medial nasal processes, which are flanked by paired lateral nasal and maxillary prominences (Feng et al., 2009). By E12.5 in the mouse, the nasal and maxillary prominences must fuse into a seam for proper development and fusion of the primary palate (Li et al., 2019). The formation of the secondary palate occurs later in development and involves vertical outgrowths from the maxillary prominences. These elevate above the tongue and fuse between E14.5 and E16.5 in the mouse (Griffith and Hay, 1992). The mechanisms of fusion in the secondary and primary palates are thought to be similar (Ray and Niswander, 2012).

The evidence for EMT in removal of the seam is controversial. It was first proposed as a fate for palatal seam cells based on early experiments observing these cells taking on a transitionary cellular phenotype *in vivo*, accompanied by upregulation of vimentin expression (Fitchett and Hay, 1989). These experiments were followed by dye labelling studies in which cells with a mesenchymal cell type were found both residing within the closed seam (Shuler et al., 1991) and as islets of mesenchymal cells dispersed in the palate mesenchyme (Shuler et al., 1992). These studies did not find evidence of apoptosis in transitioned cells (Fitchett and Hay, 1989). Using more modern technology, and the creation of a chimeric culture system using *K14cre*-labelled and wild-type palatal shelves, epithelial cells have been shown to migrate into the mesenchyme of the shelf and undergo EMT (Jin and Ding, 2006).

Other studies have tried to fate map seam cells in culture, using dyes or infection with adenovirus containing *lacZ*, and found very little evidence for EMT (Cuervo and Covarrubias, 2004). Cell-fate mapping studies using transgenic mice, specifically those tracing Shh- and K14-expressing cells, also did not provide evidence for EMT taking place, and proposed that all fusion in the palatal seam is brought about by apoptosis (Vaziri Sani et al., 2005; Xu et al., 2006). In keeping with a central role for apoptosis, knockdown of Wnt11, which is thought to regulate apoptosis in the palatal seam by inhibiting FGFR1b, results in inhibition of palatal fusion (Lee et al., 2008). However, Wnt11 can also activate several genes such as Snail1 and Zeb1 to mediate EMT (Zhang et al., 2012), so it may not exclusively mediate apoptosis during palatal seam fusion.

The involvement of apoptosis in removal of the seam is further complicated by the observation that mice mutant for caspase 3 (a key regulator of apoptosis) are still capable of primary palette fusion (Kuida et al., 1996). In addition, mice that are mutant for Apaf1, which forms part of the apoptosome, display cleft lip and/or primary palate in some mouse lines but not others (Long et al., 2013). This suggests that other cellular processes are involved in mediating the dissolution of the seam.

With advances in imaging technology, it has been possible to put forward a third hypothesis for the dissolution of the seam – convergence and extrusion (Kim et al., 2015). Studies using live imaging of the converging palate in explant cultures show establishment of contacts and intercalation between epithelial cells of the seam, which results in a transient epithelial structure. This structure is cleared as development progresses through displacement of these epithelial cells towards the oral surface through the formation of multi-cellular rosettes, which surround cells and then extrude them from the seam region (Kim et al., 2015). Other studies have also used live imaging to track the migration of these cells from the seam (Logan and Benson, 2020), revealing that the cells appear to move together as a sheet. Their ability to retain their position relative to one another but still migrate may point towards an intermediary cell state while they migrate (Logan and Benson, 2020).

TGF-β signalling plays an important role in removal of the palatal seam. TGF-β3 null mice exhibit failure in shelf fusion, allowing the shelves to be pulled apart as the head grows (Kaartinen et al., 1995). A similar phenotype is evident in epithelial-specific TGF-βr1 knockout mice (Dudas et al., 2006), and epithelial-specific TGF-βr2 knockout mice also have a cleft palate phenotype (Xu et al., 2006). In these mice, however, the failure of the seam to fuse appears to involve increased proliferation, as evidenced by the presence of epithelial cysts. This suggests that TGF-βr2 regulates both proliferation and apoptosis and mediates seam clearance through these mechanisms (Xu et al., 2006). Studies modelling seam fusion *in vitro* show cells migrating, upregulating mesenchymal-specific markers such as vimentin, followed by apoptosis, again mediated by TGF-β3 (Ahmed et al., 2007). Therefore, TGF-β3 can regulate all three possible methods for removal of the palatal seam, further clouding the field (Jalali et al., 2012; Nakajima et al., 2018; Nawshad et al., 2004; Richardson et al., 2017).

Ephrin signalling has also been implicated in fusion via EMT in the palate, independently of TGF-β signalling (Serrano et al., 2015). When cultured palates are blocked from fusing, using TGF-β3 inhibition, treatment with Ephrin signalling factors reverses this phenotype by inducing increased EMT and migration in cells of the palatal seam (Serrano et al., 2015). This demonstrates the complexity of the signalling factors controlling removal of the palatal seam and highlights that multiple signalling families are likely involved.

As mentioned above, the primary palate is formed by fusion of the medial and lateral nasal prominences with the maxillary prominence, forming a three-way seam named the lambdoidal junction (Tamarin and Boyde, 1977). Fusion mechanisms are similar to those occurring during formation of the secondary palate (Losa et al., 2018). Studies have found that those epithelial cells of the primary palate seam that do not initially undergo programmed cell death undergo EMT mediated by Smad4 and Snail1 (Losa et al., 2018). The activity of these signalling factors is, in turn, mediated by Pbx signalling, with loss of Pbx signalling resulting in cleft primary palate and lip (Losa et al., 2018). A more recent single cell RNA-sequencing analysis of primary palate fusion uncovered cell populations that undergo EMT, in addition to cell cycle arrest and migration (Li et al., 2019). This approach helps to piece together the gene interaction networks that may be at play during primary palatal fusion and that would be interesting to study in the context of secondary palate fusion, and in other tissues that undergo EMT.

The successional dental lamina

The successional dental lamina, and the breakdown of this tissue, determines the number of generations of teeth a species will have (Tucker and Fraser, 2014). One of the factors that may be involved in successional lamina breakdown is EMT (Fig. 3A).

The dental lamina is a stripe of stratified epithelia that runs along the jaw and is the tissue from which functional tooth buds initiate during development (Štembírek et al., 2010). Replacement teeth form from the successional dental lamina, which initiates after the first generation of teeth. The development, morphology and longevity of the successional dental lamina varies considerably between species (Wang et al., 2014). Most mammals, including humans and pigs, have two generations of teeth, and are classed as diphyodonts. Other vertebrates, for example reptiles such as snakes and lizards, are polyphyodonts, and display multiple generations of tooth replacement. In these animals, the successional dental lamina

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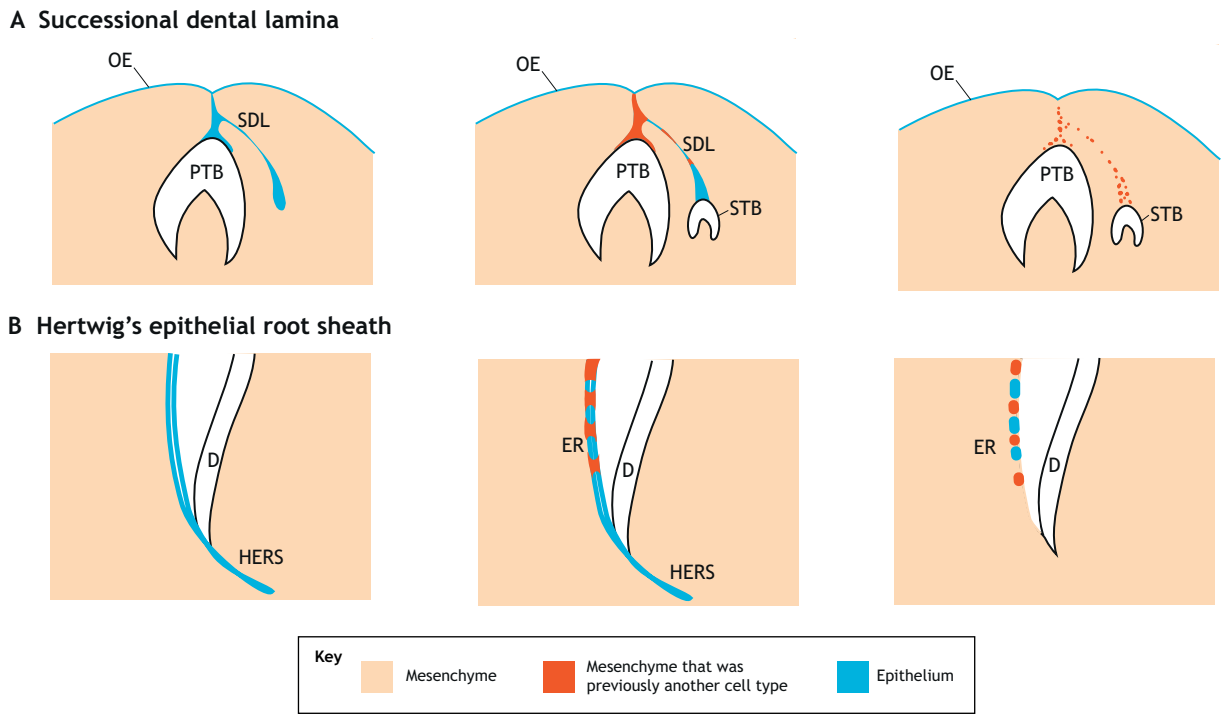

Fig. 3. EMT in the tooth. (A,B) The development of both the tooth crown and the tooth root may involve EMT. Schematics show development of successional dental lamina (A) and Hertwig's epithelial root sheath (HERS) (B). D, dentin; ER, epithelial rests; OE, oral epithelium; PTB, primary tooth bud; SDL, successional dental lamina; STB, successional tooth bud.

remains intact and continues to generate replacement teeth (Gaete and Tucker, 2013). In contrast, after the initiation of the second generation of teeth in diphyodonts, the successional dental lamina degrades, preventing further generations of teeth from developing (Buchtová et al., 2012).

Loss of the successional dental lamina in animals that cannot replace teeth throughout life could be due to several mechanisms, one of which is removal of epithelial cells through EMT, although apoptosis and migration have also been proposed (Dosedělová et al., 2015). For example, EMT, apoptosis and migration have been examined in the mini pig successional dental lamina with evidence found for all three (Buchtová et al., 2012). Evidence supporting a key role for EMT comes from the high level of E-cadherin observed in the epithelium in the early stages of tooth development. This decreases as the successional dental lamina begins to degrade (Buchtová et al., 2012). Loss of E-cadherin is associated with increasing levels of MMPs, which help to break down and restructure the ECM and promote cell motility. Snail2 protein levels also increase as the lamina degrades (Buchtová et al., 2012).

Initiation of dental lamina breakdown coincides with the presence of blood vessels surrounding the epithelium. Angiogenesis has previously been associated with EMT in tumorigenesis, and so could also play a role in dental lamina EMT (Thiery et al., 2009). In addition, regression of the dental lamina begins on the side nearest the tooth, suggesting that signalling from the tooth is important to trigger dental lamina breakdown (Buchtová et al., 2012). In agreement with this, removal of the rudimentary successional dental lamina tissue from the first tooth in the monophyodont (single generation) mouse, and subsequent culture of the tissue *in vitro* leads to an awakening of this structure, and a replacement tooth germ begins to form (Popa et al., 2019).

If the successional dental lamina does not degrade properly it can result in the development of gingival cysts such as Epstein pearls in infants (note that the term 'Epstein pearl' has also been used to describe cysts associated with the palatal seam) (Richard et al., 2000). Such cysts could be caused by failure of the migrating epithelium to successfully undergo transformation to a mesenchymal cell type (Buchtová et al., 2012). The adult disease ameloblastoma has also been proposed to result from remnants of the dental lamina (Yang et al., 2017), with epithelial cells proliferating and forming structures similar to the cap/bell stage of tooth development. Aberrant Wnt signalling and BMP expression (Heikinheimo et al., 2015), along with Sox2 and β-catenin (Fraser et al., 2019), may be a cause of the phenotype in these cells.

The breakdown of Hertwig's epithelial root sheath

Another site from which epithelial cells need to be removed is within the tooth root. Here again, EMT has been proposed (Fig. 3B).

After the completion of crown formation during tooth development, the inner and outer enamel epithelium of the tooth fuse below the crown to form a bilayered epithelium called Hertwig's epithelial root sheath (HERS) (Ten Cate, 1996). The two components of HERS are of ectodermal origin and are surrounded by the mesenchymal dental papilla (on the inside) and the dental follicle (on the outside). HERS is thought to play a key role in root development and the pattern of root bifurcation (Fons Romero et al., 2017). As the root extends, the HERS fragments to create epithelial rests (termed 'Rests of Malassez') that are scattered along the developing root (Fig. 4). Gaps between the rests allow the dental follicle to communicate directly with the forming root odontoblasts (Li et al., 2017).

Evidence from cell lines suggests that HERS cells can undergo EMT to differentiate into cementoblasts (Zeichner-David et al., 2003). In culture, HERS cells express a number of EMT markers with addition of TGF-β1 leading to a morphology change from

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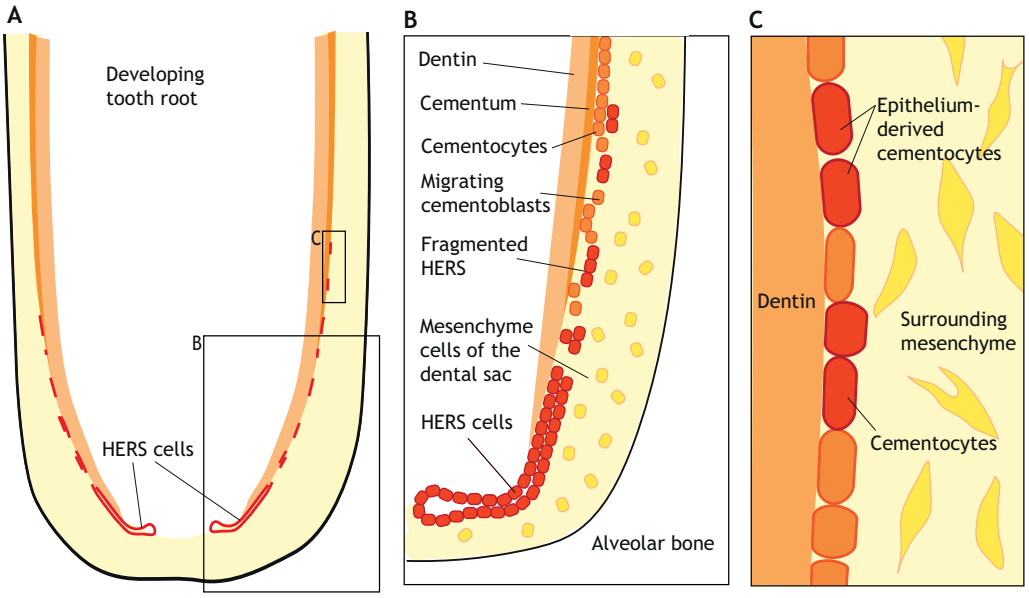

Fig. 4. EMT during breakdown of Hertwig's epithelial root sheath. (A-C) Hertwig's epithelial root sheath (HERS) is an epithelial layer that undergoes breakdown during development. This process is thought to involve EMT, allowing the HERS epithelial cell layer (red) to fragment (A). Some fragmenting HERS cells are then thought to lose their epithelial cell identity and differentiate into cementoblasts (B), becoming fully integrated into the cementocytes of the tooth root (C).

epithelial to mesenchymal (Sonoyama et al., 2007). This change is associated with a downregulation of E-cadherin and an upregulation of N-cadherin and vimentin. Transplantation of TGF-β1-treated HERS cells into mice leads to cementum formation *in vivo* (Sonoyama et al., 2007). Moreover, HERS cells established as a cell line *in vitro* are capable of expressing markers typical of both mesenchymal and epithelial cells, suggesting that they are capable of maintaining a transitionary cell phenotype (Akimoto et al., 2011). Their ability to maintain this cell type, and the ability to push the cells to a more mesenchymal cell type upon TGF-β treatment, provides further evidence that they may undergo EMT *in vivo* (Akimoto et al., 2011). Recently, organoid cultures of HERS cell lines and dental papilla cells have also been shown to develop into mineralised tissue when implanted *in vivo*, again exhibiting EMT of the HERS to form cementoblasts (Zhang et al., 2020).

Initial studies tracing the fate of HERS cells using injected traceable dyes in a mandible organ culture system found no evidence of cell migration and EMT (Yamamoto et al., 2004). However, a more recent lineage-tracing study of HERS using *K14cre;R26R* mice confirmed that some of the epithelial HERS cells lose their epithelial cell identity and differentiate into cementocytes that produce cementum (Huang et al., 2009). Although the HERS cells appear to be able to contribute to cementum, the extent to which this occurs *in vivo* appears to be quite minimal (Xie et al., 2019).

EMT in the HERS appears to be directed through TGF-β1 and FGF2 signalling via activation of the MAPK/ERK signalling pathway, with some redundancy seen between the two pathways (Chen et al., 2014). Inactivation of TGF-βR2 using an inducible knockout mouse results in disrupted molar root formation (Wang et al., 2013). Similarly, tissue-specific inactivation of Smad, an important mediator of the TGF-β signalling pathway, results in an arrest in molar root development and disruption of enamel and dentin (Huang et al., 2010). Tracing cells expressing K14 also indicates that HERS cells not only undergo EMT in order to differentiate into cementoblasts, but also migrate to contribute to the periodontal ligaments in a process also driven by TGF-β signalling (Itaya et al., 2017). As with the palate and dental lamina, a failure in EMT in HERS leads to the formation of dental cysts, which might later result in pathologies (Fraser et al., 2019).

Conclusions

EMT and its reverse, MET, are evolutionarily conserved mechanisms of differentiation (Lim and Thiery, 2012). They play vital roles in shaping the diverse and specialist structures of the vertebrate head. Many of the molecular mechanisms underlying EMT and MET are shared between different tissues, highlighting the importance of factors such as TGF-β, FGF, Snail, TWIST and ZEB. The defining features of either epithelial or mesenchymal cells, in terms of their cell-cell adhesions, polarity and ability to migrate, are also conserved across vertebrates, despite differences in the overall appearance and function of tissues. In many of these tissues, the plasticity that allows cells to transition between cell fates can also contribute to disease and can control the response to disease or injury.

Whether a neural crest cell origin allows cells to be more capable of undergoing cell-state transitions, or whether this property makes a tissue better able to repair itself after damage, is a question for future research, and almost certainly differs between cell types. Corneal endothelial cells, for example, are quiescent and expand in size rather than proliferating in response to cell death or injury (Joyce et al., 2002). However, there does appear to be a small subset of neural crest-derived progenitor cells in the corneal endothelium that are not under mitotic arrest and may be capable of contributing to repair and disease (Katikireddy et al., 2018). This property of neural crest-derived tissues, with cells under mitotic arrest interspaced with progenitor cells, is also common to other neural crest-derived tissues such as those in the heart and some neuronal tissues (Hatzistergos et al., 2015; Morrison et al., 1999). Some neural crest-derived cells appear to be especially capable of undergoing cell-state transitions, as highlighted by the ability of melanocytes to differentiate into different neural crest cell types (Vandamme and Berx, 2019). Cell-state transitions during development are certainly not restricted to neural crest-derived cells, however. For example, MET is integral to proper development of the kidney, which is derived from the intermediate mesoderm (Costantini and Kopan, 2010). Likewise, the reverse process of EMT is heavily involved in kidney fibrosis and disease (Lovisa et al., 2015).

Before the mechanisms underlying EMT/MET can be fully understood, it must first be established whether a transition has truly

taken place and what the nature of this transition is (Yang et al., 2020). To be certain that EMT/MET has taken place, multiple aspects of the cell-state transition must be assessed. This can be carried out by measuring changes in cell surface proteins characteristic of either an epithelial or mesenchymal cell type, such as cadherins, integrins and other junctional proteins. However, the analysis of surface proteins alone is not sufficient to establish that EMT has taken place (Chai et al., 2010). Assessment of cell behaviour must also be carried out, for example by examining a cell's ability to migrate and reorganise its cytoskeleton and ECM, and by assessing its polarity (Lamouille et al., 2014). The presence of transcription factors that act as regulators and inducers of EMT/MET, and their mechanisms of action, must also be ascertained (Gonzalez and Medici, 2014). In several cases of craniofacial cell-state transitions, there is some controversy over whether EMT/MET takes place or not. In the future, it may be possible to better resolve these cases by incorporating as wide a range of factors as possible in determining whether a transition has occurred. However, this issue is complicated by the realisation that some cells appear to remain in intermediate states, which makes defining when an EMT/MET takes place difficult (Sha et al., 2019).

Future research into EMT/MET will no doubt be aided by the increasing sophistication and complexity of transgenic mouse models for tracing cell fate (Skelton et al., 2013). Single cell RNA-sequencing can also now be applied to allow the trajectory of cells to be followed through development at single cell resolution (Kester and van Oudenaarden, 2018; Spanjaard et al., 2018; Vieth et al., 2019). Together, these approaches may help to resolve areas of uncertainty over the presence or mechanisms of EMT/MET in the head, such as in the middle ear and in the tooth.

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