Embryonic frog epidermis: a model for the study of cell-cell interactions in the development of mucociliary disease

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SUMMARY

Specialised epithelia such as mucociliary, secretory and transporting epithelia line all major organs, including the lung, gut and kidney. Malfunction of these epithelia is associated with many human diseases. The frog embryonic epidermis possesses mucus-secreting and multiciliated cells, and has served as an excellent model system for the biogenesis of cilia. However, ionic regulation is important for the function of all specialised epithelia and it is not clear how this is achieved in the embryonic frog epidermis. Here, we show that a third cell type develops alongside ciliated and mucus-secreting cells in the tadpole skin. These cells express high levels of ion channels and transporters; therefore, we suggest that they are analogous to ionocytes found in transporting epithelia such as the mammalian kidney. We show that frog ionocytes express the transcription factor foxi1e, which is required for the development of these cells. Depletion of ionocytes by foxi1e knockdown has detrimental effects on the development of multiciliated cells, which show fewer and aberrantly beating cilia. These results reveal a newly identified role for ionocytes and suggest that the frog embryonic skin is a model system that is particularly suited to studying the interactions of different cell types in mucociliary, as well as in secretory and transporting, epithelia.

INTRODUCTION

Mucociliary, secretory and transporting epithelia line all cavities and organs of the body, and perform a number of specialised functions. Mucociliary epithelia of the upper respiratory system provide a protective barrier against foreign particles, such as toxins, allergens and infective agents. For example, mucus in the lungs traps potentially harmful pathogens so that the cilia can move them away before they can infect the cells (Knowles and Boucher, 2002). Secretory epithelia are found in the gut, in which goblet cells secrete a protective mucus layer into the lumen to prevent infection and support the normal microflora (Specian and Oliver, 1991). The kidney can be described as a transporting system specialising in proton-secretion but also reabsorption processes, to ensure that essential molecules remain in circulation while waste products are removed (Al-Awqati and Schwartz, 2004).

There are many diseases associated with defective epithelia and they are often caused by defects in individual cell types. For example, in mucociliary epithelia, there is a range of disorders associated with aberrant ciliated cells. Primary ciliary dyskinesia (PCD) describes a number of related diseases of motile cilia, characterised by defects in the structure of the cilia and/or the ability of the cilia to beat (Eley et al., 2005). This can lead to conditions such as rhinitis and sinusitis. Defects in goblet cells can also cause mucociliary disease. Examples include chronic obstructive pulmonary disease (COPD) and asthma, in which an excess of goblet cells form. Excessive mucus production obstructs the beating of cilia required to clear pathogens, resulting in persistent and chronic infection (for a review, see Turner and Jones, 2009).

Diseases of the gut can also arise as a result of defective goblet cells. Examples include pseudomyxoma peritonei, in which an abundance of mucus is secreted as a result of increased goblet cell number, and inflammatory bowel disease, a condition linked to autoimmune production of antibodies directed against goblet cells (Ardesjo et al., 2008; O’Connell et al., 2002). Meanwhile, one of the principal roles of the kidney is to ensure acid-base homeostasis; as a consequence of defects in this function, disease often manifests itself in acidosis or alkalosis. One such example is distal renal tubular acidosis, which is caused by defects in the function of intercalated cells or indeed complete absence of these cells (Karet, 2002).

However, multiciliated epithelia and other specialised secretory epithelia such as those found in the lung, kidney and gut, are composed of several cell types that work together to form a functional organ. For example, in the mucociliary epithelia of the upper respiratory system, one finds specialised mucus-secreting goblet cells, ciliated cells, as well as serous cells (Chilvers and O’Callaghan, 2000; Fischer and Widdicombe, 2006; Houtmeyers et al., 1999). In the gut, goblet cells are also found interspersed with enteroocytes (Garcia et al., 2009), and in the kidney α- and β-intercalated cells of the collecting duct are interspersed with principal cells (Wagner et al., 2009) and multiciliated cells (Kramer-Zucker et al., 2005; Liu et al., 2007). Understanding how the breakdown in the cooperation between specialised cell types gives rise to disease has been more difficult to study than defects in single cell types.
cell types. Studying these epithelia in the whole organism is challenging because they are not easily accessible and experimental work often requires invasive techniques. Studies frequently involve fixed samples or in vitro systems that poorly recapitulate complex cell-cell interactions. Thus, there is a need for model systems that recreate the function of complex epithelia in an in vivo setting.

The frog embryonic ectoderm bears remarkable similarities with mammalian mucociliary epithelia, such as those found in respiratory tissue, because it possesses multiciliated cells and mucus-secreting goblet cells (Billett and Gould, 1971). So far, it has been used as an accessible and experimentally tractable model system to understand the molecular mechanisms of ciliogenesis (Deblandre et al., 1999; Hayes et al., 2007). It is hoped that these experiments will lead to a better understanding of the pathogenesis of diseases that involve ciliated cells (Wallingford, 2006).

Here, we expand on the studies of the frog epidermis that have looked at ciliated cells alone, to study the function of the mucociliary epithelium as a system of interacting cell types. First, we show that, alongside goblet and ciliated cells, there is a third cell type that expresses a number of ion regulators, including proton pumps such as vacuolar ATPase (v-ATPase), ion-generating enzymes such as carbonic anhydrase 12 (ca12), and ion transporters such as pendrin (slc26a4) and monocarboxylate transporter 4 (mct4). Then, we show that, like ciliated cells, ionocytes intercalate into the outer layer of the epidermis during development and their specification is under the control of the transcription factor forkhead box protein I1-ema (foxi1e). Finally, we report that the specification is under the control of the transcription factor forkhead box protein I1-ema (foxi1e). Finally, we report that the specification is under the control of the transcription factor forkhead box protein I1-ema (foxi1e).

RESULTS
Gene expression reveals the presence of ionocytes that intercalate alongside ciliated cells

The *Xenopus* larval epidermis is known to consist of mucus-secreting goblet cells and ciliated cells, bearing multiple beating cilia (Billett and Gould, 1971). However, double staining with a ciliated-cell marker and a goblet-cell marker revealed the presence of additional cells that are found in almost equal numbers as the ciliated cells (Fig. 1A, arrow) but their molecular profile and physiological properties are not known. Previous studies have reported a population of uncharacterised intercalating non-ciliated cells (INCs) that intercalate in an irregular pattern (Stubbs et al., 2006) (Fig. 1B). Such an uncharacterised population has also been shown, or inferred, in previous studies as developing alongside mucus-secreting goblet cells (Billett and Gould, 1971; Hayes et al., 2007; Mitchell et al., 2009; Montorzi et al., 2000; Nickells et al., 1988; Stubbs et al., 2006).

To gain a better understanding of these cells, we pulled genes that showed an irregular spotted expression pattern in the embryonic epidermis from a previous in situ expression screen. Most of the genes that fit this criterion were found to encode ion channels (such as subunits of v-atpase; referred to as atp6v), enzymes (such as ca12) and transporters (such as slc26a4 and mct4) (Fig. 1C). V-ATPase is a large multimeric enzyme that uses energy from ATP hydrolysis to actively pump out protons. Together these subunits act to acidify both the local environment and intracellular vesicles, in many organs. For example, in the kidney, V-ATPases are important for final urinary acidification and bicarbonate (HCO3−) reabsorption (Jouret et al., 2005; Wagner et al., 2004). We have examined the expression of subunits atp6v1a (herein referred to as v1a), atp6v1g (referred to as v1g) and atp6v0d (referred to as v0d); all showed a spotted and irregular pattern within the epidermis (Fig. 1C).

*Ca12* encodes for a transmembrane enzyme that catalyses the reversible hydration of carbon-dioxide-releasing protons and HCO3−. This enzyme has been shown to be expressed in secretory cells of the kidney and stomach of mouse and human (Kallio et al., 2006; Liao et al., 2009b), as well as being upregulated in tumours (Proescholdt et al., 2005). *ca12* showed similar expression to *v1a* in the *Xenopus* epidermis, but was also expressed in the otic and olfactory placodes (Fig. 1C). Slc26a4 is a chloride (Cl−)-HCO3− exchanger found to be involved in renal chloride reabsorption (Wagner, 2007), whereas Mct4 exchanges lactate for protons, particularly in glycolytically active cells (Dimmer et al., 2000). Both of these proteins displayed a scattered, spotted distribution but mct4 also showed expression in the somites (Fig. 1C). To find out whether the cells expressing these were intercalating cells, we sectioned embryos at early neurula stages and early tailbud stages following fluorescent in situ hybridisation for *ca12* and *v1a*. The change in gene expression can be seen in transverse sections (Fig. 1D). The spotted pattern changed from the inner layer (at early neurula; stage 14) to the outer layer (at early tailbud; stage 25) over time (Fig. 1D), suggesting that these cells undergo intercalation during approximately the same time period as ciliated cells and INCs (Stubbs et al., 2006).

These data suggest that the INCs represent a cell type that is rich in ion modulators and is likely to have a role in regulating the ionic homeostasis in the epidermis. Such cells are found in the fish epidermis and gills, where they are called ionocytes (Hwang, 2009). By analogy, we propose that these cells in the frog epidermis are also termed ionocytes.

Immunohistochemistry and ultrastructure of *Xenopus* embryonic epidermis reveals two types of ionocytes

Immunofluorescence experiments in whole mounts and sections conclusively showed that ionocytes, that is, v1a- and ca12-expressing cells, are distinct from ciliated cells, which are marked with acetylated α-tubulin (Fig. 2A-F). In most cases, ionocytes were closely associated with ciliated cells, such that most ciliated cells make direct contact with one or two ionocytes. In some cases, ionocytes developed next to each other. There were more v1a-expressing cells than ca12-expressing cells, suggesting the existence of different ionocyte subtypes, as will be further discussed below. ca12 was localised to the basolateral side (Fig. 2F) but two subcellular localisations of v1a were observed; in some v1a-positive cells, v1a was apically localised (type 1; Fig. 2C,G,H,J), whereas in the majority it was basolaterally localised (type 2; Fig. 2G,I,J). Finally, on the basis of double immunostaining with anti-v1a and anti-ca12, we suggest that there are at least two subtypes of ionocyte; one that coexpresses...
v1a and ca12, and one that expresses v1a but not ca12 (Fig. 2K-M).
Furthermore, in the cells that coexpress v1a and ca12, v1a is basolaterally localised, whereas in the cells that express v1a only, it is apically enriched. In the fish and adult frog, at least two subtypes of ionocytes have been described (Ehrenfeld and Klein, 1997; Horng et al., 2009; Hwang, 2009; Liao et al., 2009a) and, similarly, two types of intercalated cells, \( \alpha \) and \( \beta \), are found in the mammalian kidney (Brown and Breton, 1996). Apical localisation of v1a is consistent with what is observed in \( \alpha \)-intercalated cells in the mammalian kidney (Brown et al., 1981; Kyllonen et al., 2003).

To gain a better understanding of the ultrastructure of the cell types found in the outer layer of embryonic Xenopus epidermis, we performed scanning (SEM) and transmission (TEM) electron microscopy on stage 30 wild-type embryos (Fig. 3). We were able to obtain evidence of intercalating cells (Fig. 3A,B). These cells had a triangular shape and inserted themselves by extending a cytoplasmic process towards cell adhesion junctions (Fig. 3B). Goblet cells were flatter and characterised by numerous vesicles that were densely arrayed below the apical membrane, occasionally releasing their content to the outside (Fig. 3C,D). These vesicles are relatively small (1.26±0.07 \( \mu \)m) and contain granular material shown to be positive for the epidermal lectin, xeel (Fig. 3C,D) (Nagata, 2005), which is also called intelectin-2 (Hayes et al., 2007). In addition to xeel, the material being secreted might also contain mucus and, indeed, in some EM images, a layer of material coating the surface of the embryo could be seen (data not shown). To date, there is little information as to which mucins are secreted by these goblet cells.

Ciliated cells were characterised by possessing numerous cilia that were docked just below the apical membrane by a basal body and that connected to the internal cytoskeleton by a rootlet, as previously described (Fox and Hamilton, 1971; Park et al., 2008; Yang and Li,
Another population of cells seemed to possess apical microvilli and these also tended to be triangular in shape, developed in close apposition to ciliated cells and sometimes appeared in pairs (Fig. 3G,H). A population of cells with apical pits could also be seen in SEM (Fig. 3I,J). The apical openings differ in size (0.64-1.3 μm) between cells, but this might indicate different phases of secretory activity. Both microvilli-bearing cells and apical-pit-bearing cells had an abundance of mitochondria (evident for microvilli-rich cells in Fig. 3G) and they might correspond to the ion-modulating cells. In zebrafish and in adult frog skin, mitochondria-rich cells (MR cells) are so-called because they have an abundance of mitochondria that are believed to energise the ionic activity of the cell (Ehrenfeld, 1998; Esaki et al., 2007). They are most probably the same cell type as ionocytes. Although EM analysis confirmed the presence of additional cell types to ciliated and goblet cells in the embryonic frog skin, immuno-EM will be needed to accurately correlate these to the cell types identified by in situ hybridisation and immunohistochemistry.

**foxi1e is expressed in ionocytes and controls the expression of v1a**

To find out whether ionocytes have a role in the development of the epidermis, first we sought to find out how their formation is regulated. We cloned and characterised the transcription factor foxi1e in *Xenopus tropicalis*, because members of this family have been shown to be important in ionocyte development in zebrafish and other systems. In *Xenopus laevis*, the closest homologue is foxi1e (also known as xema) (supplementary material Fig. S1). Earlier studies in the frog showed that the expression of foxi1e is controlled by interaction of vegT, bmp and notch signalling, and, in turn, foxi1e is needed to form both neural and epidermal ectoderm (Mir et al., 2008; Mir et al., 2007; Suri et al., 2005). foxi1e was shown to be expressed in scattered cells in the epidermis that were distinct from ciliated cells; however, the expression was not characterised further (Mir et al., 2008; Mir et al., 2007; Suri et al., 2005). In the *X. tropicalis* tadpole, foxi1e was also found to be expressed in a scattered pattern (Fig. 4A). Cross-section analysis showed that foxi1e-expressing cells intercalate from the inner to the outer layer of the epidermis, suggesting that they are either ciliated cells or ionocytes (Fig. 4A).

Immunohistochemistry using an anti-foxi1e antibody together with anti-acetylated α-tubulin or anti-xeel showed that the foxi1e-expressing cells do not overlap with either ciliated or goblet cells, suggesting that they are likely to be ionocytes (Fig. 4B). Indeed, combining in situ probes with immunohistochemistry showed that
all foxi1e cells expressed v1a. However, only 70% of foxi1e-positive cells expressed ca12. Direct comparison between v1a and ca12 confirmed that all ca12-positive cells express v1a but not all v1a-positive cells express ca12 (Fig. 4C; see also Fig. 2M). The simplest explanation for this result is that both ionocyte subtypes (ca12+/v1a+ and ca12–/v1a+) express foxi1e. In zebrafish, foxi3a and foxi3b are expressed in different subtypes of ionocytes, whereas the mammalian Foxi1 is present in intercalated cells in the kidney (Kurth et al., 2006), the inner ear (Hulander et al., 2003) and the epididymis (Blomqvist et al., 2006), sites where ionic homeostasis is crucial for function.

We then investigated whether foxi1e is sufficient to induce the development of ionocytes. Overexpression of foxi1e by mRNA injection in one cell of the two-cell stage embryo increased the number of cells expressing v1a on the injected side of neurula and tadpole stage embryos (Fig. 5A,B). Combining v1a in situ hybridisation with anti-acetylated α-tubulin immunohistochemistry showed that there...
was an excess of v1a-positive cells on the injected side and that these formed at the expense of ciliated cells (Fig. 5B). Thus, foxi1e is not only a marker of ionocytes but it also specifies their formation.

**foxi1e knockdown depletes the population of ionocytes in the epidermis**

foxi1e was knocked down using either a translation-blocking morpholino (ATG MO) that covered the ATG start site or a splice MO (sp MO) that covered the splice acceptor site (Fig. 6A). The efficiency of the splice MO was confirmed by the aberrant pattern of splicing (Fig. 6B). Ornithine decarbocylase (odc) is a ubiquitously expressed gene that was used as a loading control to show that loss of foxi1e was due to the presence of the splice morpholino rather than a difference in the amount of starting material. The efficiency of the ATG MO was shown by both the reduction of foxi1e-haemagglutinin (HA) protein level in the embryo (supplementary material Fig. S2) and reduced immunostaining for foxi1e (Fig. 6D). Both MOs gave the same phenotypic results, confirming the specificity of the phenotype.

By comparing the in situ expression patterns of v1a and α-tubulin in embryos injected with the foxi1e ATG MO, sp MO or an MO control (MOC), it was clear that loss of foxi1e leads to loss or reduction in expression of v1a, whereas α-tubulin expression was not affected (Fig. 6C). It was unclear whether the ion-modulating cells were still present in the epidermis but did not express v1a or whether this cell type was missing altogether. To clarify this, we performed in situ hybridisation for foxi1e transcript, after foxi1e protein had been knocked down with the ATG morpholino (Fig. 6B), reasoning that the loss of ionocytes due to foxi1e depletion would be evident by a loss in foxi1e mRNA expression. Indeed, the level of foxi1e mRNA was reduced substantially in the epidermis, suggesting that ion-modulating cells are absent (Fig. 6D). This was also supported by two more observations. First, there were no cells in the epidermis that were neither α-tubulin nor xeel positive (Fig. 6D). Second, in the MO-injected embryos the pattern of goblet cells around the ciliated cells assumed a rosette formation (Fig. 6D), which reflects the regular pattern of intercalation of ciliated cells. Normally, this pattern is broken by the irregular intercalation of non-ciliated cells (Stubbs et al., 2006), which we now know are ionocytes. The persistence of this rosette pattern in the epidermis of MO-injected embryos indicates that ionocytes are lost, leaving just goblet and ciliated cells.
**Loss of ionocytes has a detrimental effect on the mucociliary epidermis**

To look more closely at the surface of embryos lacking ionocytes, we immunostained for acetylated α-tubulin and ca12 or v1α. As expected, ca12 and v1α were also missing at the protein level (Fig. 6E). Although α-tubulin expression was not affected in the foxi1e morphants (Fig. 6C), the immunostaining for acetylated α-tubulin was reduced, raising the possibility that the differentiation of ciliated cells is abnormal. When foxi1e was knocked down, each ciliated cell had fewer cilia, which seemed shorter and sometimes entwined with each other (Fig. 6E). This experiment was repeated independently four times and these defective cilia were apparent and significantly different than control embryos (Student’s t-test: P<0.01, MOC=2/32 embryos with defective cilia; foxi1e ATG=42/47 embryos with defect; Fig. 6E).

Embryos injected with foxi1e MO did not ‘drift’ in the culture dish, a characteristic behaviour due to the beating of cilia, and fluorescent beads moved considerably slower over the surface of morphant embryos, suggesting abnormal ciliary beating (data not shown).

To gain a greater insight into the impact of foxi1e knockdown on the mucociliary epidermis as a whole, SEM and TEM were performed on morphant embryos. These showed that small cells with apical openings were absent from the surface. Just as shown by immunofluorescence, the SEM images indicated that the major defect was in the ciliated cells (Fig. 7A,B). The cilia are less abundant and seem to be shorter than in control embryos. TEM images showed that the ciliated cells in the foxi1e morphant embryos possessed numerous basal bodies, but these were abnormally located deep in the cytoplasm (Fig. 7C, marked by arrowheads). By contrast, in the controls, basal bodies were found close to the apical membrane, where they normally dock (Park et al., 2008). Thus, it is very likely that the reduction in the number of cilia is due to failure of basal bodies to migrate and/or dock to the apical side. There were also some noticeable changes in the morphology of the cells surrounding the ciliated cells. These were more difficult to describe but it seems that these cells have developed excessive villi or protrusions. Indeed, TEM images also showed that, in experimental animals, goblet cells harboured cytoplasmic protrusions resembling microvilli, which seemed covered by a dense material (Fig. 7D, arrowheads). In addition, goblet cells were larger compared with controls (also seen by xereal staining, Fig. 6D).

**Ionocytes exert a non-cell-autonomous effect on ciliated cells**

Our results so far showed that the loss of ionocytes impacts negatively on the maturation and function of the ciliated cells, suggesting that there is a non-cell-autonomous effect from the ionocytes to the neighbouring ciliated cells. To test this idea further, we transplanted foxi1e MO-injected ectoderm (FITC labelled) into a wild-type host, to form a morphant clone of cells for analysis at the tadpole stage (mosaic analysis; Fig. 8A). As expected, the morphant clone showed lack of ionocytes and had abnormal ciliated cells, with fewer cilia. We did not detect abnormal ciliated cells in the wild-type tissue adjacent to the morphant clone, most probably owing to the abundance of wild-type ionocytes. However, normal ciliated cells were found close to the border of the morphant tissue, indicating a rescue of ciliated cells by their wild-type neighbours. Mingling of normal and morphant epidermal cells was observed at the borders of the clone; however, the rescued ciliated cells were clearly labelled with the FITC-tagged MO and were found next to wild-type ionocytes.

**DISCUSSION**

The embryonic epidermis of *Xenopus* is characterised by the presence of multiciliated cells and, as such, it has been used as a
Fig. 6. Impact of foxi1e knockdown on the mucociliary epidermis. (A) Diagrammatic representation of foxi1e, showing the position of the ATG MO (red) and sp MO (blue). E1, exon 1; E2, exon 2; I, intron. (B) Reverse transcriptase (RT)-PCR with ornithine decarboxylase (odc) control and foxi1e primers, showing that abnormal splicing of foxi1e is observed when a foxi1e sp MO is injected. Asterisk marks reduction in foxi1e splice product in foxi1e splice morphants compared with control. (C) Injection of foxi1e sp MO reduces (11/11) and injection of foxi1e ATG MO abolishes (11/11) the expression of v1a, whereas MOC has no effect (0/9) at early tadpole stage as shown by whole-mount in situ hybridisation. Neither foxi1e sp MO (0/13), foxi1e ATG MO (0/11) or MOC (0/10) had an effect on expression of the ciliated cell marker α-1-tubulin. Scale bars: 500 μm. (D) Embryos were injected with MOC or foxi1e ATG MO (20 ng each) and analysed as indicated. In situ hybridisation with foxi1e probe shows that the spotted expression of foxi1e is reduced (9/9) in foxi1e morphants, whereas MOC has no effect (0/11). Scale bars: 500 μm. Immunostaining of tadpole epidermis with anti-fox1e antibody shows that the protein expression is reduced in foxi1e morphants (11/12) but not in the MOC controls (0/10). In situ hybridisation with α-1-tubulin probe (black), combined with anti-xeel antibody staining (red), as indicated, reveals that, in the foxi1e morphants, the number of cells that are not positive for either α-1-tubulin or xeel is greatly reduced compared with controls. This suggests that the missing cells are ionocytes. Actin staining (phallolidin–Alexa-Fluor-488; green) combined with anti-acetylated α-tubulin antibody (red) reveals that epidermal cells are arranged in a rosette pattern around each ciliated cell in the foxi1e-ATG-MO-injected epidermis, but in the MOC control (stained with anti-ZO-1 and acetylated α-tubulin), this rosette formation is broken by the insertion of ionocytes (inset shows higher-magnification view). See text for details. Scale bars: 50 μm. (E) Immunostaining for v1a (green), ca12 (green) and acetylated α-tubulin (red) in the combinations indicated confirms that ionocyte markers are missing in foxi1e-ATG-MO-injected embryos (34/35; n=5 experiments) but not in MOC controls (0/35; n=5 experiments). Scale bars: 50 μm. High-magnification images (zoom; scale bar: 20 μm) reveal that ciliated cells are abnormal in the foxi1e morphants. Bar graph shows quantification of the experiments looking at defective ciliated cells (n=4 experiments). Defective ciliated cells were evident in the majority of foxi1e ATG morphant embryos (42/47), but not in MOC-treated embryos (2/32). **P=0.0018.
model system to elucidate the cell-autonomous molecular and cellular mechanisms by which cilia are formed and beat in a single, polarised direction (Mitchell et al., 2007; Park et al., 2008). Here, we show the existence of an additional population of cells in the *Xenopus* embryonic epidermis; these cells express proton pumps such as v-atpase subunits, enzymes such as ca12, and ion exchangers such as slc26a4 and mct4. These cells do not coincide with either ciliated or mucus-secreting goblet cells but, like the ciliated cells, undergo intercalation from the inner into the outer layer of the epidermis. Therefore, we suggest that the previously uncharacterised INCs (Stubbs et al., 2006) are most probably analogous to the ionocytes found in zebrafish epidermis and gills, where they play a role in osmotic regulation and clearance of metabolic by-products (Horng et al., 2009; Esaki et al., 2009). Ionocytes have also been called MR or proton-secreting cells in both adult frogs and fish embryos (Ehrenfeld and Klein, 1997; Lin et al., 2006), and can also be found in the mammalian kidney, where they have been termed intercalated cells (Brown and Breton, 1996). In zebrafish skin and gills, ionocytes energise the uptake of ions such as Na⁺ and Cl⁻ against their natural concentration gradients in freshwater environments. They do this by using energy from ATP hydrolysis to pump out protons through V-ATPases and electrogenically drive the intake of Na⁺ ions. Because *Xenopus* embryos must also survive in similar freshwater conditions to zebrafish, it is likely that the *Xenopus* larval ionocytes have similar ionic regulation roles. The requirement for ionic regulation continues in the adult and, indeed, ionocyte-like cells have been described in adult frog skin, which has been used as a model for epithelial V-ATPase-dependent Na⁺ transport for many years (Ehrenfeld, 1998; Ehrenfeld and Klein, 1997).

Here, we show that, in addition to osmotic regulation, ionocytes have a role in the correct function of the mucociliary epithelium. Ionocytes express the winged helix transcription factor foxi1e, and knocking down foxi1e results in depletion of ionocytes. Although this is in contrast to previous studies in *X. laevis* that have concluded that knockdown of foxi1e leads to loss of ciliated cells (Mir et al., 2007), it is consistent with the previous observation that foxi1e morphants eventually die from osmotic lysis (Mir et al., 2007), and with the results in zebrafish (Esaki et al., 2009; Hsiao et al., 2007; Jänicke et al., 2007) and mice (Blomqvist et al., 2006; Hulander et al., 2003; Kurth et al., 2006; Vidarsson et al., 2009) on the regulation of ion-modulating genes by Foxi1 in renal, epididymal and endolymphatic duct/sac epithelium. In mice, loss of FOXI1 causes deafness, infertility and distal renal tubular acidosis (Blomqvist et al., 2004; Blomqvist et al., 2006; Hulander et al., 2003;
consistent with a loss of ionocytes (Blomqvist et al., 2004).

What is the impact of ionocyte loss on the development of the frog larval mucociliary epidermis? In foxi1e morphants, ciliated cells develop aberrantly in that they possess fewer and shorter cilia. This causes a reduction of acetylated $\alpha$-tubulin staining, which might have led authors of previous studies to erroneously suggest that ciliated cells have been lost in foxi1e morphants (Mir et al., 2007). Aberrant cilia are also unable to beat properly: embryos did not show usual drifting motion, which is characteristic of cooperative epidermal cilia beating (not shown). TEM analysis showed that basal bodies fail to localise to the apical side, where they would normally dock. Because foxi1e is not expressed in ciliated cells, we conclude that the effect on ciliated cells is secondary to the loss of ionocytes. This is further supported by loss-of-function mosaic experiments that showed that morphant abnormal ciliated cells could be rescued by wild-type ionocytes. But how could ionocyte loss account for defects in the ciliated cells?

Loss of ionocytes, with consequent loss of ion-modulating pumps and channels, could affect the development of ciliated cells by altering the ionic properties of the local environment. In turn, this could affect the structure and/or secretion of mucus from adjacent goblet cells. In support of this, there is evidence that, in mammals, ionic regulation by enterocytes is important for mucus release from goblet cells (De Lisle, 2009; Garcia et al., 2009). In particular, HCO$_3^-$ forms complexes with Ca$^{2+}$ and H$^+$, and it is the dissociation of these cations from mucins that enables mucin expansion and disaggregation (Garcia et al., 2009; Quinton, 2008). Enterocytes express the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl$^-$ channel that has been shown to also regulate HCO$_3^-$ transport (Choi et al., 2001). In cystic fibrosis, which is believed to arise as a result of mutations in this Cl$^-$-HCO$_3^-$ channel (De Braekeleer and Daigneault, 1992), defective secretion of HCO$_3^-$ by enterocytes leads to defects in mucus secretion by the goblet cells, resulting in abnormally thick, aggregated, mucus (Garcia et al., 2009). In the lung, such abnormally thick mucus impedes mucociliary clearance and could secondarily damage the ciliated cells, which are unable to clear it (Houtmeyers et al., 1999; Voyer, 2009).

Alternatively, recent studies have shown that acidification by V-ATPase is important for the activation of several signalling pathways that are involved in embryonic development, such as Wnt, Notch and Fz-Dsh (Buechling et al., 2010; Cruciat et al., 2010; Vaccari et
It is intriguing that the ciliary defects observed following the loss of ionocytes are very similar to those observed from loss of Dsh in ciliated cells, including shortened cilia and abnormal basal body migration and docking (Park et al., 2008). Thus, it could be that an ion modulator such as V-ATPase is necessary for a signalling event that activates Dsh in the ciliated cell. Our mosaic analysis suggests that the ionocyte–ciliated-cell signal has a very short range and might even be cell-contact dependent. Loss of ionocytes could also affect the internal pH of the ciliated cell, because a recent paper suggests that docking of Dsh is affected by changes in intracellular pH (Simons et al., 2009). However, we have no further evidence, at present, to link the foxi1e morphant phenotype to loss of dsh signalling in the ciliated cell.

In the foxi1e morphant, the goblet cells were still present but we were not able to determine whether mucus secretion or expansion was normal, because the mucins secreted by the goblet cells have not yet been characterised in *Xenopus*. For this reason, at present, we cannot distinguish whether the non-cell-autonomous effects are direct on the ciliated cells or indirect via the goblet cells. Although these possibilities are not mutually exclusive, we favour a direct interaction between ciliated cells and ionocytes for two reasons. First, ionocytes tend to develop in physical contact with ciliated cells and, second, the defects in cilia seem to have an intracellular aetiology (failure of basal body migration and/or docking), which would be difficult to explain by altered mucus properties. Taken together, our results show that the loss of ionocytes leads to defects in the composition and function of the mucociliary epithelium.

To conclude, our work showed for the first time the impact of ionocytes on ciliated cells using an in vivo system. Our work also expanded the usefulness of the *Xenopus* larval skin as a model system for epithelial development. The larval epidermis is easily accessible and is amenable to live imaging and molecular manipulations, but so far its use has been restricted to the study of ciliated cells. The embryonic zebrafish skin, and the adult frog skin, had been used as models of transporting epithelia because neither possesses ciliated cells [in the frog, ciliated cells disappear after hatching (Nishikawa et al., 1992)]. Our work showed that the embryonic frog skin is more versatile as an epithelial in vivo model system than previously thought, because it consists of secretory, ion-modulating and ciliated cells. Thus, it can be used to study secretory and transport as well as mucociliary epithelia. We suggest that this system would be best exploited to understand how different cells interact with each other to form a functional organ. Furthermore, it can be used to understand not only how disease states can arise by defects in a single cell type but also how it can arise as a result of the breakdown in cooperation between different cell types.

**METHODS**

**Morpholino design**

All morpholinos were designed based on the *X. tropicalis* genome sequence and were purchased from Gene Tools. The *foxi1e* gene sequence has two exons either side of an intron, and gives rise to mRNA of 1122 nucleotides and an expected protein sequence of 373 amino acids (41 kDa). The sequence of the *foxi1e* ATG MO is 5’-GTGCTCTGTGGATCAATGCACCTCAT-3’, of the sp MO is 5’-AATGAATGGTCTTACCTGGATCAT-3’ and of the standard MO control (mutated β-globin gene) is 5’-CCTCTTACCTCAT-AGTTACAATTTATA-3’.

**Constructs and mRNA generation**

A pCS2-foxi1e-HA construct was generated by PCR amplification of the full-length gene, linearised downstream of the HA tag with the enzyme NotI (Roche) and transcribed with the SP6 RNA polymerase. mRNA was injected into two-cell stage embryos at a dose of 250 pg with lacZ RNA (250 pg) as a lineage marker. To generate probes for in situ hybridisation, specific ESTs were obtained from the *X. tropicalis* full-length database. The clone names were as follows: v-atpase subunit αd (TNeu100k18), v-atpase subunit VIa (TTpa014f09), v-atpase subunit vIG (TTpa033b12), mct4 (Tegg054k10), slc26a4 (TTba078m21), ca12 (TEgg122a02) and foxi1e (TNeu069d21). Where appropriate, gene names were adjusted according to standard nomenclature (www.xenbase.org).

**Whole-mount in situ hybridisation**

In situ hybridisation was performed largely as described (Harland, 1991). Embryos were post-fixed in methanol and bleached in 5% hydrogen peroxide (H2O2). To perform antibody staining after in situ hybridisation, the proteinase K step was omitted from the protocol. After development of the colour reaction for in situ hybridisation, two 30-minute washes in methanol were performed followed by stepwise rehydration into BBT [PBS-Triton (0.1% Triton X-100) + 5% BSA]. The standard immunofluorescence protocol was then followed as below.

For fluorescent in situ hybridisation, the following modifications were made to the standard protocol: after post-hybridisation washes, endogenous peroxidase activity was inactivated by incubating with 3% H2O2 in PBS for 20 minutes. The antibody used to detect the probes was anti-Dig-POD (1:1000; Roche) and signal was detected using Cy-3-based tyramide signal amplification (TSA kit; Perkin Elmer), as described previously (Vize et al., 2009). To stain nuclei, DAPI (5 mg/ml) was incubated with the embryos for 5 minutes.

**Immunofluorescence**

Antibody staining was carried out on whole-mount embryos according to standard protocols. In summary, embryos were fixed in MEMFA [0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% (v/v) formaldehyde] and post-fixed in methanol. All antibodies used in immunofluorescence were used at a dilution of 1:1000. The antibodies used were mouse anti-acetylated α-tubulin (Sigma), rabbit anti-ca12 [generated by Cambridge Research Biochemicals (CRB)], rabbit anti-foxi1e (generated by CRB), mouse anti-xcel (gift from Saburo Nagata, Japan’s Women’s University, Tokyo, Japan), rabbit anti-via1 v-atpase (named ST170; gift from Shigeyasu Tanaka, Shizuoka University, Japan), rat anti-via1 v-atpase (gift from Shigehisa Hirose, Tokyo Institute of Technology, Japan) and rabbit anti-ZO-1 (used at a dilution of 1:200; Zymed). For staining of actin, phalloidin conjugated to Alexa Fluor 488 was used (1:300 dilution; Invitrogen) and combined with antibody staining for acetylated α-tubulin. After hourly washes with BBT, secondary antibodies conjugated to

MO control (mutated β-globin gene) is 5’-CCTCTTACCTCAT-AGTTACAATTTATA-3’.
Alexa Fluor 488, Alexa Fluor 568 and Alexa Fluor 647 (1:500 dilution; Molecular Probes, Invitrogen) were used according to the species of primary antibody being recognised. Secondary antibodies were also incubated overnight with the embryos at 4°C. The embryos were imaged in glass-bottom dishes (MatTek Corporation) using a confocal microscope (Olympus Fluoview; FV-1000). Imaging settings were kept the same for control and experimental embryos.

**Cryosectioning**

Embryos were sectioned after whole-mount in situ hybridisation or immunofluorescence. Pre-neurula stage embryos were embedded in 15% fish gelatin (Sigma). Post-neurula stage embryos were embedded in 25% fish gelatin. The embedded embryos were frozen in fish gelatin prior to sectioning. Sections were 12 μm thick. After sectioning, slides were mounted in Mowiol (Polysciences; for immunofluorescence) or 90% glycerol (for in situ samples) and coverslips sealed over sections for imaging.

**Antibody generation**

An antibody for ca12 was generated from a portion of the protein (residues 21-155) found to be unique among other carbonic anhydrases. This portion was cloned into the pMal-c2x vector (NEB) so that it was tagged to maltose-binding protein (MBP) for immunisation into rabbits. The crude extract was tested and was positive for expression of ca12 by immunofluorescence. The anti-fox1e antibody was generated from a peptide sequence: LGNSSPGTDDSSEKRSPP. This peptide was synthesised and used to immunise rabbits. The crude extract was positive in immunofluorescence and the antibody was affinity-purified against the peptide. Both antibodies were generated by CRB.

**Western blot**

Embryos injected at the one-cell stage were grown until gastrula stage (stage 10), frozen and lysed in lysis buffer [150 mM NaCl, 20 mM NaAc, 1% NP-40, 5 mM EDTA, 5 mM EGTA + 1 mM PMSF and complete protease inhibitors (Roche)]. The lysate was run on a 10% SDS-PAGE gel. Western blotting was performed in a semi-dry system according to manufacturer’s instructions (Hoefer). Prior to antibody incubation the polyvinylidene fluoride membrane (Millipore) was blocked with 5% milk powder in PBS-Tween (0.1%, v/v) for 1 hour. For HA signal, rat anti-HA peroxidase (1:1000; Roche) was used. For the tubulin loading control, mouse anti-tubulin primary antibody (1:10,000; Sigma) was used. Antibodies were incubated in blocking buffer overnight, followed by washes with PBS-Tween. Blots were developed using ECL reagents (GE Healthcare).

**Transplant assay**

Embryos injected with foxie ATG MO were reared alongside wild-type embryos until gastrulation. Embryos were incubated in transplantation buffer [0.5× MMR, 1% ficoll and 5 μg/ml gentamicin (Sigma)] throughout the transplantation procedure. Forceps were used to transplant a small patch of epidermal tissue from the ventral animal pole of the morphant embryos onto the wild-type embryos (with ventral tissue removed). The embryos were then incubated in post-transplantation buffer (0.1× MMR, 5 μg/ml gentamicin) until stage 30, when they were fixed for analysis. For diagrammatic representation see Fig. 8A.

**Electron microscopy**

SEM and TEM were performed according to standard protocols. 70-nm sections were cut on an Reichert-Jung Ultracut E ultramicrotome and observed using an FEI Tecnai 12 electron microscope at 80 kV.

**ACKNOWLEDGEMENTS**

The authors thank the staff in the EM facility in the Faculty of Life Sciences, University of Manchester, for their assistance, and the Wellcome Trust for equipment grant support to the EM facility. They thank Aleksand Mironov for expert technical support with transmission electron microscopy, Leslie Lockey for assistance with the preparation of samples for scanning electron microscopy and Patrick Hill for use of the scanning electron microscope. They also thank Chris

**TRANSLATIONAL IMPACT**

**Clinical issue**

Complex epithelia have multiple functions, including the regulation of ion transport and homeostasis. A failure to regulate ion homeostasis is a hallmark of many human diseases. For example, defects in the function of epithelia whose primary function is ion transport, such as those found in kidney and epididymis, lead to abnormalities such as distal renal acidosis and male sterility. In addition, although ion transport is not the primary function of other epithelia, such as the secretory and mucociliary epithelia found in the gut and lung, ionic regulation is crucial for their normal function. In diseases such as cystic fibrosis, failure of ionic regulation is associated with abnormalities in mucus consistency and secretion, which has deleterious effects in the gut and lungs.

Most epithelia consist of several cell types, including specialised cells dedicated to ionic regulation (known as ionocytes, mitochondria-rich cells, proton-secreting cells or intercalated cells). Challenges in understanding disorders of epithelial ion homeostasis include understanding how the different cell types cooperate to form a functional organ and how abnormalities in one cell type can affect the function of other cell types. However, owing to the difficulties in studying epithelia in vivo in mammals, there is an urgent need for model systems that recapitulate the complexity of the epithelia yet still enable experimental observation and manipulation.

**Results**

The frog embryonic (larval) skin, which contains both mucus-secreting goblet cells and multiciliated cells, has been used as a model system for mucociliary epithelia of the lung, particularly to understand the molecular mechanisms of ciliogenesis. Here, the authors show that the epidermis of Xenopus tropicalis larvae also contains a population of ionocytes in addition to mucus-secreting goblet cells and multiciliated cells. They show that the ionocytes express proteins that modulate ion homeostasis, such as v-atpase, carbonic anhydrase 12, pendrin and monocarboxylate transporter 4, and intercalate from the inner to the outer layer of the epidermis. Furthermore, X. tropicalis ionocyte development depends on the transcription factor foxi1e, indicating that they are highly similar to the intercalated cells found in the mammalian kidney. Knockdown of foxi1e expression prevents the development of ionocytes, which, surprisingly, has a deleterious effect on the development of multiciliated cells, which show fewer and aberrantly beating cilia.

**Implications and future directions**

This work demonstrates for the first time the influence of ionocytes on ciliated cells and multiciliated cells, and expands the utility of Xenopus larval skin as a versatile model for the study of secretory, transporting and mucociliary epithelia in vivo. Combined with other advantages of Xenopus as a model (amenability to live imaging and manipulation of gene expression, as well as a fully sequenced genome), this represents a powerful system in which to examine how different cell types interact to form a functional organ, to model complex epithelial diseases and to test potential therapies.

doi:10.1242/dmm.007195
Kintner for communicating results prior to publication, and Saburo Nagata, Shigeyasu Tanaka and Shigehisa Hirose for the donation of antibodies. N.P. is a Wellcome Trust Senior Research Fellow and E.D. is a Wellcome Trust 4-year PhD student. This work was supported by the Wellcome Trust.

COMPETING INTERESTS
The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS
E.D. and N.P. designed the experiments and wrote the manuscript; E.D. performed the experiments and N.P. directed and supervised the work.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.006494/-/DC1

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