BMP signalling controls the construction of vertebrate mucociliary epithelia

Marie Cibois1,4, Guillaume Luxardi1,4,‡, Benoît Chevalier2,‡, Virginie Thomé1, Olivier Mercéy2,3, Laure-Emmanuelle Zaragosi2,3, Pascal Barbrý2, Andrea Pasini1,§, Brice Marcet2,3,§ and Laurent Kodjabachian1,§,¶

ABSTRACT

Despite the importance of mucociliary epithelia in animal physiology, the mechanisms controlling their establishment are poorly understood. Using the developing *Xenopus* epidermis and regenerating human upper airways, we reveal the importance of BMP signalling for the construction of vertebrate mucociliary epithelia. In *Xenopus*, attenuation of BMP activity is necessary for the specification of multiciliated cells (MCCs), ionocytes and small secretory cells (SSCs). Conversely, BMP activity is required for the proper differentiation of goblet cells. Our data suggest that the BMP and Notch pathways interact to control fate choices in the developing epidermis. Unexpectedly, BMP activity is also necessary for the insertion of MCCs, ionocytes and SSCs into the surface epithelium. In human, BMP inhibition also strongly stimulates the formation of MCCs in normal and pathological (cystic fibrosis) airway samples, whereas BMP overactivation has the opposite effect. This work identifies the BMP pathway as a key regulator of vertebrate mucociliary epitheliun differentiation and morphogenesis.

KEY WORDS: Xenopus, Human, Mucociliary epithelium, Multiciliogenesis, BMP, Notch, Epidermis, Airway

INTRODUCTION

Mucociliary epithelia (MCE) are encountered in many bilaterians, where they perform a variety of functions, including animal movement, food particle capture and ingestion, gamete transportation, and protection against pollutants and infectious agents (Castillo-Briceno and Kodjabachian, 2014; Lyons et al., 2006; Rompolas et al., 2013; Silverman et al., 1999; Werner and Mitchell, 2012). One of the most widely studied MCE is the lining of the human upper airways, which ensures the clearance of inhaled noxious particles by means of the mucociliary escalator. Several chronic respiratory diseases, including asthma, chronic obstructive pulmonary diseases and cystic fibrosis, are associated with defects in MCE regeneration and impaired airway cleansing (Fahy and Dickey, 2010; Livraghi and Randell, 2007). More recently, familial cases of deficient mucociliary clearance have been linked to mutations in genes required for multiciliogenesis (Boon et al., 2007; Hogan et al., 2014; Rocket et al., 2009). The cellular and molecular events controlling human airway MCE formation and renewal are incompletely understood, largely owing to the poor accessibility and experimental amenability of this tissue. Air-liquid interface (ALI) primary cultures of human airway epithelial cells (HAECs) from healthy or diseased donors provide a valuable tool to investigate these processes (Karp et al., 2002; Marcet et al., 2011), but are technically demanding and might not fully recapitulate the relevant interactions occurring within the organism.

Recent studies have suggested that the embryonic epidermis of the amphibian *Xenopus* might provide a powerful and valuable model to address vertebrate MCE biology (Cibois et al., 2014; Dubaissi and Papalopulu, 2011; Werner and Mitchell, 2012). In particular, several reports revealed a striking degree of conservation in the molecular mechanisms that control MCC differentiation in mammalian upper airways and in the *Xenopus* embryonic epidermis (Marcet et al., 2011; Song et al., 2014; Stubbs et al., 2012; Tan et al., 2013; Werner and Mitchell, 2012).

In its mature form, the *Xenopus* embryonic epidermis consists of an epithelial outer layer of goblet cells that is interspersed with MCCs, osmoregulatory ionocytes and serotonin-secreting small secretory cells (SSCs), and an inner layer of non-cohesive P63-positive cells (Deblandre et al., 1999; Dubaissi et al., 2014; Lu et al., 2001; Quigley et al., 2011; Walentek et al., 2014). This tissue emerges from the early non-neural ectoderm in a multistep process. During gastrulation, MCCs, ionocytes, SSCs and P63-positive cells are born within the inner layer. It has been reported that the Delta/Notch pathway controls the number of cells of each type, presumably via lateral inhibition. In this process, a so-called signal-sending cell displays ligand molecules, such as Delta, at its surface and transactivates Notch receptor molecules on the surface of adjacent signal-receiving cells, which allows the two cells to adopt distinct fates (Guruharsha et al., 2012). In the *Xenopus* epidermis, constitutive activation of Notch resulted in a decrease in the number of both MCCs and ionocytes, whereas blocking the Notch pathway led to an increase in their numbers (Deblandre et al., 1999; Hayes et al., 2007; Marcet et al., 2011; Quigley et al., 2011; Stubbs et al., 2006, 2012). However, there is evidence that *Xenopus* epidermal cells are sensitive to cis-inhibition of Notch by its ligands (Guruharsha et al., 2012), as Delta-like 1 (Dll1) overexpression induces supernumerary MCCs, similar to overexpression of the secreted dominant-negative Dll1Smu mutant (Deblandre et al., 1999). Finally, Notch activation was shown to induce supernumerary P63-positive cells, whereas Notch inhibition had the opposite effect, suggesting that P63-positive cells are counterparts to MCCs and ionocytes selected through lateral inhibition (Sirour et al., 2011). Following their commitment,
MCCs, ionocytes and SSCs sequentially migrate to the outer layer, where they intercalate among goblet cells and resume differentiation (Chung et al., 2014; Cibois et al., 2014; Quigley et al., 2011; Stubbs et al., 2006), whereas the P63-positive cells remain in the inner layer. The role of P63-positive cells remains uncharacterised, although the mature inner layer of the amphibian embryonic epidermis has been proposed to constitute the source of the late larval and post-metamorphic skin (Yoshizato, 2007). Thus, *Xenopus* epidermal P63-positive cells may not be compared to the P63-positive bona fide basal stem cells of mammalian airway epithelia (Hogan et al., 2014).

In *Xenopus*, BMP signalling is chiefly responsible for non-neural ectoderm induction at blastula/gastrula stages (Cibois et al., 2014; De Robertis and Kuroda, 2004). Although BMP expression and activity remain high at subsequent stages (Schohl and Fagotto, 2002), the role of this pathway in the developing epidermis has never been addressed. In mammals, activation of the BMP pathway has been described in lung morphogenesis and homeostasis as well as in airway epithelium regeneration (Huang et al., 2014; Masterson et al., 2011; McCormack et al., 2013; Sountoulidis et al., 2012). We show here that correct organogenesis of the *Xenopus* embryonic epidermis requires the fine-tuning of BMP signalling activity. BMP inhibition is necessary and sufficient to induce MCC, ionocyte and SSC specification, whereas the P63-positive cells remain in the inner layer. Furthermore, in HAECs from healthy donors and from cystic fibrosis patients, BMP pathway inhibition stimulates MCE differentiation, with a dramatic increase in MCC numbers, whereas treatment with recombinant BMPs globally blocks cell differentiation.

Altogether, our data highlight the importance of the BMP pathway in the construction of vertebrate MCE.

**RESULTS**

**The BMP pathway is active during MCE development and regeneration**

Phosphorylation of Smad1/5/8 (pSmad1/5/8), which indicates BMP activity, was detected in the nuclei of most non-neural ectoderm cells in *Xenopus*, in both the outer and inner ectodermal layers and at all stages analysed, ranging from blastula to tailbud (Fig. 1A). To map the cell populations that receive BMP signals in the inner layer, we performed pSmad1/5/8 immunodetection in embryos injected with the reporter constructs α-tub::GFP or pendrin::GFP, which drive GFP expression in committed MCCs and ionocytes, respectively (Quigley et al., 2011; Stubbs et al., 2006). As shown in Fig. 1B, pSmad1/5/8 immunoreactivity was detected in both MCCs and ionocytes before and after their intercalation into the outer layer. In addition, co-immunostaining for P63 (Tp63–Xenbase) showed that some pSmad1/5/8-positive inner layer cells were P63 positive (Fig. 1B), revealing that the BMP pathway was active in at least three out of the four currently described cell types born within the inner layer.

In regenerating HAECS, pSMAD1/5 levels increased between the proliferation and polarisation stages, then decreased at the onset of ciliogenesis (Fig. 2A). Although at the polarisation stage the pSMAD1/5 signal was stronger in the apical cell layer of the pluristratified epithelium (Fig. 2B), it increased in the basal layer following treatment with recombinant BMP2 (Fig. 2C,D), showing that basal layer cells were also responsive to the BMP signal.

Thus, BMP pathway activation during the early steps of MCE formation is a conserved feature in our two models. We next addressed the consequences of BMP pathway dysregulation for MCE formation.
BMP pathway overactivation perturbs the construction of Xenopus epidermal and human airway MCE

Injection of stage 9 Xenopus embryos with recombinant BMP4 led to increased Smad1/5/8 phosphorylation and nuclear localisation (supplementary material Fig. S1A,B). Scanning electron microscopy (SEM) of the mature epidermis of BMP4-injected embryos revealed a striking decrease in the number of MCCs, ionocytes and SSCs, whereas goblet cells were still apparent (Fig. 3A,A''). In situ hybridisation or immunostaining with markers specific for MCCs (\(\alpha\)-tubulin and acetylated tubulin; Fig. 3B-C''), ionocytes (foxi1e and v1a; Fig. 3C-D'') and SSCs (tph1 and serotonin; Fig. 3E-F'') confirmed the reduction in the numbers of these cell types. Overactivation of the BMP pathway by injection of a constitutively active (CA) form of the BMP receptor Alk3 (Bmpr1a – Xenbase) in ventral ectoderm also led to a decrease in the numbers of MCCs and ionocytes (supplementary material Fig. S2A-C''). Surprisingly, a loss of P63 immunoreactivity was observed in BMP-treated embryos (Fig. 3G,G''), suggesting that non-intercalating inner cells were also affected. By contrast, BMP4 treatment did not significantly alter the expression of otogelin and trim29 (Fig. 3H-J''), and rather upregulated intelexin-1 expression (Fig. 3G,G''), suggesting that goblet cell identity was maintained.

Although the epidermis occasionally appeared thicker in sections of BMP-injected embryos, immunostaining against phosphohistone H3 did not reveal any consistent variation in the number of mitotic nuclei (supplementary material Fig. S2F-H). We next tested whether the observed lack of differentiation of intercalating cells resulted from an earlier defect in specification. For this, we analysed how BMP treatment affected the expression, prior to intercalation, of foxj1, foxi1e and foxa1, which label committed MCCs, ionocytes and SSCs, respectively. As shown in Fig. 3K-M'', BMP4 injection at stage 9 resulted in a drastic decrease in the early expression levels of these three markers, revealing a defect in the specification of inner intercalating cell types.

In HAECs, chronic treatment with recombinant BMP2 strongly reduced the number of MUC5AC-positive goblet cells, both at polarisation and late ciliogenesis stages (Fig. 4A-B). Likewise, BMP2 treatment suppressed MCCs, as revealed by the absence of acetylated tubulin-positive cells at the late ciliogenesis stage (Fig. 4A-B). Following BMP2 treatment, the cells presented a flattened aspect, reminiscent of the morphology observed in squamous metaplasia, a common alteration of the human upper airway lining (data not shown). Consistent with this interpretation, BMP2-treated HAECs exhibited increased levels of transglutaminase 1 (TGM1) and involucrin (IVL), two markers of squamous metaplasia (Gray et al., 2007; Tanabe et al., 2012) (Fig. 4C).

We conclude that BMP pathway overactivation is incompatible with the construction of a normal MCE in both models. We next addressed the consequences of BMP inhibition in this process.

BMP pathway inhibition perturbs the construction of Xenopus epidermal and human airway MCE

Inhibition of the endogenous BMP pathway in the Xenopus non-neural ectoderm was achieved by knocking down BMP2, BMP4 and BMP7 with specific morpholino oligonucleotides (BMP MOs), as previously reported (Reversade et al., 2005). In all subsequent
experiments, BMP MOs were injected into 8-cell stage animal ventral blastomeres that are fated to become epidermis, together with membrane GFP (mGFP) RNA to visualise and count injected cells. We first verified that BMP MOs suppressed Smad1/5/8 phosphorylation in injected cells (supplementary material Fig. S1C). BMP MO injection resulted in an increase in the numbers of inner layer cells expressing early markers of MCCs (foxj1 and multicilin/MCI; Fig. 5A-′B′) and ionocytes (foxi1e; Fig. 5C-′C′). A corresponding increase in the number of committed MCCs and ionocytes was visible at tailbud stage (Fig. 5G-′I′,P,Q). Injection in the presumptive epidermis of synthetic mRNAs encoding dominant-negative (dn) forms of the BMP receptor Alk3 or the Smad5 transcriptional effector also resulted in an expansion of the MCC and ionocyte populations (supplementary material Fig. S2A″-E). BMP knockdown also caused a significant expansion of SSCs marked early by foxa1 (Fig. 5D,D′) and in stage 35 tadpoles by tph1 (Fig. 5R and Fig. 6I-J′).

Unexpectedly, transverse sections through BMP morphant tailbud embryos revealed that α-tubulin-positive cells remained trapped between the two layers of the epidermis and failed to produce cilia (Fig. 6A-C′,F-H′,K-M′). The failure of MCCs to intercalate was not simply due to a developmental delay, as most remained trapped below the surface layer in late tadpoles (Fig. 6F-H′,K-M′). MCCs also displayed incomplete intercalation in dnSmad5-injected embryos, although this defect was not as pronounced as in BMP morphants (supplementary material Fig. S1E). An analogous failure to intercalate was observed for ionocytes and SSCs in stage 25 and stage 35 BMP morphants, respectively (Fig. 6D-E′,I-J′).

Finally, BMP MO injection also resulted in lower levels of expression of the goblet cell markers otogelin and trim29 at stage 14 (Fig. 5E-F′), as well as of the 5G7 antigen and intelectin-1 at stage 25 (Fig. 5J-L′). No statistically significant variation in the number of P63-positive non-intercalating inner cells was observed (Fig. 5S), but these cells displayed lower levels of P63 and α-dystroglycan (Fig. 5M-O′).
In HAECs, BMP inhibition was achieved through chronic treatment with recombinant Noggin protein, a potent secreted BMP antagonist, or with the pharmacological BMP pathway inhibitor dorsomorphin (Yu et al., 2008). We first verified that Noggin could suppress BMP-induced SMAD1/5 phosphorylation and nuclear translocation (supplementary material Fig. S3A,B). Noggin treatment led to a dramatic dose-dependent increase in the number of MCCs (Fig. 7A,B,D-F; supplementary material Fig. S3C). A similar induction was seen upon dorsomorphin treatment (Fig. 7C,F). Fluorescence-activated cell sorting (FACS) confirmed that the number of acetylated tubulin-positive MCCs and MUC5AC-positive goblet cells was increased by ~4-fold by Noggin (Fig. 7G). The effect of Noggin was strongest when treatment started around the polarisation stage, when BMP pathway activation was maximal (Fig. 2A), and a single 3-day-long pulse of Noggin was sufficient to massively stimulate MCC differentiation (Fig. 7H).

In cystic fibrosis (CF), chronic injuries of the airways lead to epithelium remodelling that is characterised by mucous secretory hyper/metaplasia and a progressive loss of MCCs, dramatically impairing mucociliary clearance and airway defence (Livraghi and Randell, 2007). Thus, we examined whether BMP pathway inhibition by Noggin treatment was also capable of inducing an increase in the number of MCCs in primary cultures derived from CF patients. Fig. 7I-K shows that the effect of Noggin was maintained in CF primary cultures, suggesting that BMP pathway inhibition might stimulate MCC formation and improve mucociliary clearance in chronic airway disease patients.

The BMP and Delta/Notch pathways are linked in the *Xenopus* epidermis

Since the Delta/Notch pathway controls the numbers of MCCs and ionocytes in the *Xenopus* epidermis (Deblandre et al., 1999; Hayes et al., 2007; Quigley et al., 2011; Stubbs et al., 2006), we speculated that the BMP signal might act through this pathway. Injection of recombinant BMP4 into blastula stage 9 embryos resulted in a strong and persistent upregulation of the Notch ligand *dll1* throughout the ectodermal inner layer (Fig. 8A-D,H-I). Conversely, when presumptive epidermal blastomeres were injected with GFP mRNA together with either dnSmad5 mRNA or BMP MOs, a consistent cell-autonomous repression of *dll1* expression was observed compared with embryos injected with GFP mRNA alone (Fig. 8E-G′; data not shown). This was accompanied by an increase in *dll1* expression in some adjacent uninjected cells, most likely owing to reduced activation of the Notch pathway in these cells by the injected cells that contained less Dll1 ligand (Fig. 8E-G′). Strikingly, the ability of exogenous BMP to upregulate *dll1* transcription was temporally limited and coincided with its capacity to suppress MCC, ionocyte and SSC fates. Indeed, as shown in Fig. 8H-J″, blastocoel injection of BMP4 at stage 9 resulted in both *dll1* upregulation at stage 12 and loss of *α*-tubulin at stage 25, whereas both markers were unaffected when the injection was performed at gastrula stage 11. Interestingly, BMP4 injection at stage 11 did not prevent the normally specified inner cells from reaching the superficial layer (data not shown).

To confirm that the control of *dll1* expression by the BMP pathway was compatible with the observed effect of BMP overexpression on MCCs, ionocytes and SSCs, we examined *dll1* expression relative to early markers of these three cell types. We found that, during gastrulation, *dll1* was first co-expressed with the MCC marker *foxj1*, and soon after with the ionocyte marker *foxi1e* (Fig. 8K-R). This is consistent with the comparable effects produced by Notch pathway activation and repression on the number of both MCCs and ionocytes (Deblandre et al., 1999; Hayes et al., 2007; Quigley et al., 2011; Stubbs et al., 2006). Co-expression of *dll1* and the early SSC marker *foxal* was also observed at early neurula stage 14 (Fig. 8S-V), consistent with the repression of *foxal*...
Fig. 5. BMP inhibition promotes MCC, ionocyte and SSC fates in the developing Xenopus epidermis. (A–O’) Eight-cell stage Xenopus embryos were injected in one animal ventral blastomere (fated to become only epidermis) with either 500 pg GFP mRNA alone (Cntl) or with 500 pg GFP mRNA and BMP2, BMP4 and BMP7 morpholinos (BMP MOs, 10 ng each) and were analysed at stage 14 (A–F’) or 25 (G–O’). GFP immunostaining was used to identify the injected cells. Injection of BMP MOs resulted in an increase in the numbers of stage 14 inner layer cells expressing markers for committed MCCs (foxj1 and MCI, red in A,A’ and B,B’, respectively), for committed ionocytes (foxi1e, red in C,C’) and for committed SSCs (foxa1, red in D,D’). Conversely, injection of BMP MOs led to a severe decrease in the expression levels of the goblet cell markers otogelin and trim29 (red in E,E’ and F,F’, respectively). When analysed at stage 25, embryos injected with BMP MOs showed an increase in the numbers of α-tubulin-positive MCCs (white in G,G’ and H,H’) and foxi1e-positive ionocytes (red in I,I’). Conversely, injection of BMP MOs led to a severe decrease in the expression levels of the outer layer goblet cell markers intelectin-1 (red in J,J’,K,K’) and 5G7 (white in L,L’) and of the inner layer non-intercalating cell markers P63 (white in M,M’,O,O’) and α-dystroglycan (red in N,N’,O,O’). (A–F’,M–O’) Cryosectioned embryos; (G–L’) Whole-mount embryos. (P–S) Quantification of the different inner layer cellular populations in injected epidermal clones at stage 25. Shown are the percentages of MCCs (P), ionocytes (Q), SSCs (R) and P63-positive inner non-intercalating cells (S) among injected, GFP-positive cells. The increase in the number of MCCs, ionocytes and SSCs in BMP morphants was significant (Student’s t-test). No significant variation was observed for P63-positive cells. Error bars indicate s.d.
expression in Notch intracellular domain (NICD)-injected embryos (Hayes et al., 2007).

In summary, tightly regulated BMP activity appears to be required for dll1 expression and the specification of MCCs, ionocytes, SSCs and non-intercalating inner cells, presumably through the Notch pathway. Consistent with this view, injection of a dominant-negative form of the Notch effector Su(H) was able to limit the decrease in MCC specification caused by a constitutively active form of the BMP receptor, while a constitutively active form of Su(H) was able to counteract the increase in MCC specification produced by a dominant-negative form of the BMP receptor (supplementary material Fig. S4).

**DISCUSSION**

Our study reveals that the construction of MCE in two distant vertebrate models commonly involves the BMP signalling pathway. Below, we highlight similarities and differences between the responses to BMP modulation in our two models.

In *Xenopus* epidermis, we found that exogenous BMP4 prevents the specification of MCCs, ionocytes, SSCs and P63-positive non-intercalating inner cells. In other words, all inner cell fates are suppressed when BMP is over-active. By contrast, outer layer goblet cells are specified normally. Conversely, BMP pathway inhibition leads to an increase in the numbers of MCCs, ionocytes and SSCs, but not P63-positive cells, and antagonises goblet cell differentiation. In HAECs, both goblet and MCC fates are suppressed by exposure to exogenous BMP2 protein, and both fates are induced by BMP inhibition. Thus, goblet cells exhibit opposite responses to BMP in our two systems. This is likely to reflect the difference in the goblet cell lineage in the two models. In HAECs, goblet cells and MCCs are likely to derive from common P63-positive progenitors (Hogan et al., 2014), and it seems logical that they exhibit similar responses to BMP modulation. In the *Xenopus* epidermis, goblet cells are born in the outer layer whereas MCCs are born in the inner layer. The two layers of the epidermis are produced through oriented cell divisions during cleavage stages and inherit different maternal determinants that control inner and outer cell fates (Chalmers et al., 2003; Ossipova et al., 2007). Thus, goblet cells and MCCs are born from lineages that have been separated before the activation of the zygotic genome, which might explain why they respond in an opposite manner to the zygotic inducer BMP. The *Xenopus* embryonic epidermis also contains ionocytes, which are involved in osmoregulation (Dubaisi and Papalopulu, 2011; Quigley et al., 2011), and SSCs, which control the ciliary beating frequency of MCCs and secrete anti-infective substances that protect the embryo (Dubaisi et al., 2014; Walentek et al., 2014). Ionocytes and SSCs have no clear counterparts in human airways, so no pertinent comparison can be made.

The most striking parallel between our two models is the identical response of MCCs to BMP pathway modulation. This observation is consistent with multiple reports of common molecular mechanisms at the basis of MCC differentiation in vertebrates (Boon et al., 2014; Marcet et al., 2011; Song et al., 2014; Stubbs et al., 2012; Tan et al., 2013).
fates, but rather promotes in the non-neural ectoderm a permissive and P63-positive cells. Thus, the BMP signal might not instruct cell inner layer cells, which will give rise to MCCs, ionocytes, SSCs that the BMP signal exerts its action on an early pool of multipotent (Snape et al., 1987; Wylie et al., 1987). This observation indicates competence of embryonic cells to respond to exogenous inducers exogenous BMP4 ends by mid-gastrula stage 11. Interestingly, this window of susceptibility of the developing timing or strength of the signal cannot be ruled out. The temporal pathway activity, although cell type-specific differences in the the developing epidermis, all cell types appear to experience BMP subsequently to be tightly controlled to ensure MCE formation. In et al., 2014; Mou et al., 2012; Sountoulidis et al., 2012), has Robertis, 2006) and for lung morphogenesis in mammals (Huang 2013; Wallmeier et al., 2014). We conclude that, at the present time, the comparison between HAEC cultures and the developing Xenopus embryonic epidermis is mostly relevant to an understanding of MCC biology. Recent studies have developed protocols to generate in vitro airway epithelial cells from human pluripotent stem cells that include an early inhibition of the BMP pathway followed by its activation in order to push the definitive endoderm to differentiate into ventral anterior foregut, before the induction of lung progenitor specification (Firth et al., 2014; Huang et al., 2015, 2014). However, these reports did not explore the role of BMP signalling at later steps, when airway progenitors give rise to fully differentiated airway MCE. Our work reveals that BMP inhibition may facilitate the commitment of multipotent airway progenitors towards MCC and goblet cell fates, making it an important signalling pathway to be considered for human airway regeneration in physiological as well as pathological situations.

The activity of the BMP pathway, which is initially required for the partitioning of the non-neural ectoderm in Xenopus (De Robertis, 2006) and for lung morphogenesis in mammals (Huang et al., 2014; Mou et al., 2012; Sountoulidis et al., 2012), has subsequently to be tightly controlled to ensure MCE formation. In the developing epidermis, all cell types appear to experience BMP pathway activity, although cell type-specific differences in the timing or strength of the signal cannot be ruled out. The temporal window of susceptibility of the developing Xenopus ectoderm to exogenous BMP4 ends by mid-gastrula stage 11. Interestingly, this stage is known to mark the end of the temporal window of competence of embryonic cells to respond to exogenous inducers (Snape et al., 1987; Wylie et al., 1987). This observation indicates that the BMP signal exerts its action on an early pool of multipotent inner layer cells, which will give rise to MCCs, ionocytes, SSCs and P63-positive cells. Thus, the BMP signal might not instruct cell fates, but rather promotes in the non-neural ectoderm a permissive state compatible with fate choices by downstream regulators. In HAEC cultures, the BMP treatment almost completely obliterates the formation of both MCCs and goblet cells and results in the expression of markers of squamous epithelia. This is reminiscent of the squamous metaplasia that occurs when the airway epithelium is submitted to chronic damage or irritation and might reflect the excessively prolonged maintenance of the cells in an uncommitted state (Hogan et al., 2014). Altogether, our data suggest that, in developing or regenerating vertebrate MCE, fate commitment cannot be initiated when BMP activity is too high. We propose that attenuation of BMP activity, by as yet unknown mechanisms, is required for cells to engage in fate choices.

We found that BMP signalling is required to activate dll1 expression in the Xenopus developing epidermis, although the absence of clear Smad consensus binding sites upstream of the dll1 open reading frame (data not shown) suggests an indirect mode of control. The decreased dll1 expression in the absence of BMP is expected to reduce Notch activation and allow a greater number of cells to engage in intercalating cell fate choices. In agreement with this interpretation, Dll1 knockdown induces supernumerary MCCs (Marcet et al., 2011). By contrast, the strong and persistent induction of dll1 expression in BMP4-injected embryos was correlated with the lack of specification of all inner cell types. This finding is at odds with the published observation that injection of a synthetic dll1 mRNA leads to an increase in the number of MCCs, presumably through cis-inhibition of Notch (Deblandre et al., 1999). Thus, Notch cis-inhibition by increased levels of dll1 transcripts might not occur in the presence of excess BMP activity. Conversely, increased dll1 expression in BMP4-injected embryos might not translate into Notch activation either, as it should otherwise induce P63 expression (Sirour et al., 2011). We conclude that BMP overactivation produces inhibitory effects that make it impossible for inner layer cells to initiate their specification programme. Such inhibitory effects might include the artificial
maintenance of pluripotency regulators by excess BMP activity (Morrison and Brickman, 2006; Scerbo et al., 2012; Ying et al., 2003). However, the inhibition caused by BMP may be overcome, to a certain extent, by the Notch pathway, as suggested by the antagonism observed when the BMP and Notch pathways were concomitantly manipulated in opposite ways (supplementary material Fig. S4).

Following their specification in the inner epidermal layer, the MCCs, ionocytes and SSCs migrate to the outer layer, where they intercalate among goblet cells, a morphogenetic step crucial for the development of the functional Xenopus epidermis, but which has no clear counterpart in the regenerating HAEC cultures. Our data show that blocking the BMP signal by injection of BMP MOs completely and durably prevents intercalation of MCCs, ionocytes and SSCs. The failure in intercalation might depend non-exclusively on a cell-autonomous disruption of cytoskeleton dynamics in intercalating cells, or on defects in the differentiation of the inner and/or outer layer cells that render the epidermal environment non-permissive for intercalation. Although this issue deserves further investigation, it is remarkable that the transmembrane protein α-Dystroglycan, which is expressed by inner non-intercalating cells and is downregulated following BMP knockdown, has been shown to be required for MCC intercalation (Sirour et al., 2011).

Thus, in the Xenopus epidermal MCE, BMP activity coordinates cell fate specification with cell movement. It is important to stress that this dual role was not reported for the Notch pathway, as supernumerary MCCs induced by Notch inhibition in the epidermis do manage to intercalate (Deblandre et al., 1999; Stubbs et al., 2006).

In conclusion, our study reveals that vertebrate MCE construction involves the BMP pathway at multiple steps of the organogenetic process. Beyond the global overview provided by this study, more focused analyses will be required to understand how BMP activity is spatially and temporally controlled, to identify at the molecular level the responses induced by BMP modulation, and to decipher the complex interplay with other signalling pathways.
MATeRiALS AND MeTHODS

Human tissue samples
Inferior turbinates or nasal polyps were from patients who underwent surgical intervention for nasal obstruction or septoplasty (kindly provided by Prof. Castillo, Pasteur Hospital, Nice, France). Samples from CF patients were purchased from Epithelial Sarl (Geneva, Switzerland). The use of human tissues was authorised by biosafety law 94-654 of the French Public Health Code after written consent from the patients.

Ethics statement
All experiments were performed following the European Directive 2010/63/EU on the protection of animals used for scientific purposes. All animal experiments were approved by the ‘Direction de l’Environnement des Populations, Pôle Alimentation, Santé Animale, Environnement, des Bouches du Rhône’ (agreement number E 13-055-21).

Isolation and culture of human airway epithelial cells
Primary HAEC cultures were performed according to Marcat et al. (2011). HAEC differentiation was analysed at four time points following exposure of HAECs at an air-liquid interface (ALI). Pr, Po, EC and LC represent the proliferating step at ALI day 0, the polarisation step at ALI day 7, the early multiciliogenesis step at ALI day 14 and the late multiciliogenesis step at ALI day 21, respectively.

General Xenopus procedures
Eggs obtained from NASCO females were fertilised in vitro, dejellied, cultured and injected as described previously (Marchal et al., 2009). Synthetic capped mRNAs were produced with the Ambion mMESSAGE mACHINE Kit. BMP2, BMP4 and BMP7 morpholinos were described by Reversade et al. (2005). Recombinant zebrafish Bmp4 protein was resuspended as recommended by the manufacturer (R&D Systems, catalogue number 1128-BM), and injected through the animal pole into the blastocoelic cavity of embryos at blastula or at gastrula stages. Plasmids for the MCC α-tub::GFP and ionocyte pendrin::GFP reporter constructs were linearised by SfiI and injected into one animal cell at the 8-cell stage.

Stainings

Xenopus
Whole-mount chromogenic in situ hybridisation was performed as described previously (Marchal et al., 2009). Whole-mount fluorescent in situ hybridisation (FISH) was performed as described previously (Castillo-Briceno and Kodjabachian, 2014). For single staining, all RNA probes were hybridised by incubation in increasing sucrose concentrations and finally embedded in LR White. After FISH, the embryos were embedded in LR White and sectioned (PFA), stored in methanol for at least 4 h at −20°C, then rehydrated in PB (PBS+Tween 0.1% v/v), treated with triethanolamine and acetic anhydride, incubated in increasing sucrose concentrations and finally embedded with OCT (VWR Chemicals). 12-μm-thick cryosections were created. FISH on sections was an adaptation of the whole-mount FISH method. Immunohistochemical staining was performed on whole embryos as described previously (Castillo-Briceno and Kodjabachian, 2014) and adapted for sections.

Human
Fresh cultures of ALI-D28 (LC) HAECs were used for immunodetection as previously described (Marcat et al., 2011). Cells were fixed (4% PFA, 15 min, 4°C), rinsed (0.1 M glycine in PBS, 10 min) and permeabilised (0.1% Triton X-100, 5 min). Fixed cells were blocked for 1 h in 3% BSA, and incubated for 1 h at room temperature or overnight at 4°C with the appropriate primary antibodies (supplementary material Table S1). Then, cells were incubated for 1 h with the appropriate secondary antibodies (supplementary material Table S1). Stained cells were mounted with ProLong Gold antifade reagent (Invitrogen, Life Technologies). After FISH and IHC, and just before mounting, samples were placed in DAPI (1 μg/ml in PBS) for 3 min for whole Xenopus embryos and HAECs and 2 min for sections.

Imaging
Images of HAEC cultures were acquired using an Olympus Fv10i or Leica SP5 confocal imaging system with 60× oil-immersion objective. Epidermal tissue from Xenopus embryos was explanted and mounted with Fluormount G (Fluorprobes) and allowed to dry before imaging on a Zeiss LSM780 confocal microscope. Images were acquired as 8 bit/channel and with 1024×1024 pixel resolution, and processed in ImageJ for maximum intensity z-projection and/or merge of channels. Expression levels on FISH were analysed using ImageJ. For Xenopus, stacks of confocal images from four to five examps per experiment and per condition were made. Z-projection of the green channel was used to count GFP-positive injected cells. MCCs, ionocytes and SSCs were counted using a merge of their corresponding channels with the green channel on order to consider only injected cells. Statistical analysis was made using GraphPad Prism 6.

SEM processing and imaging

Xenopus
Samples were prepared and imaged as previously described (Castillo-Briceno and Kodjabachian, 2014).

Human
SEM was performed at the CCMA EM Core Facility of the University of Nice Sophia-Antipolis. Briefly, cells were fixed in 1.6% glutaraldehyde in 0.1 M phosphate buffer, rinsed and post-fixed 30 min in osmium tetroxide (1% in 0.1 M phosphate buffer). After rinsing, cells were dehydrated in a graded ethanol series and dried using hexamethyldisilazane (HMDS). Cells were mounted on aluminum stubs with adhesive tabs, sputter-coated with Pt (Cressington, 308R) and examined on a 6700F field emission scanning electron microscope (JEOL).

Western blot
Primary HAECs were harvested by scraping in RIPA lysis buffer (Thermo Scientific Pierce) and cleared by centrifugation. Protein concentration was determined using the BCA assay (Thermo Fisher Scientific) and equivalent amounts of protein were resolved by electrophoresis using the Novex NuPAGE SDS-PAGE Gel System following the manufacturer’s instructions. Proteins were transferred to PVDF membranes (Bio-Rad) and analysed by immunoblotting with appropriate primary antibodies (supplementary material Table S1) and HRP-conjugated secondary antibodies (1/5000, Dako). Immunoreactive bands were detected using the Immobilon ECL Kit (Merck Millipore) on an LAS-3000 imager (Fujifilm).

Acknowledgements
We thank the staff of the imaging platform and of the aquatic facility at IBDM; Jean-Pierre Lauzier (Centre Commun de Microscopie Appliquée, University of Nice Sophia-Antipolis) for the SEM experiments on human samples; Chris Kintner, Nancy Papalopulu, Martin Blum, John Wallingford, Eric Bellefroid and Saburo Nagata for sharing reagents; and Pierluigi Scerbo, who made the initial observation of misexpression of epidermal cell type marker genes in response to BMP pathway dysregulation in Xenopus. The authors declare no competing or financial interests.

Author contributions
M.C., G.L. and V.T. performed experiments on Xenopus and analysed data. B.C., O.M., L.-E.Z. and B.M. performed experiments on HAEcs and analysed data. A.P. and B.M. drafted the article and L.K. edited it. L.K. and B.M. conceived and supervised the project. L.K. and P.B. obtained funding and supervised the research teams. L.K. coordinated the project.

Funding
This work was supported by Centre National de la Recherche Scientifique (CNRS), Aix-Marseille Université, Université de Nice Sophia-Antipolis, and by grants from the Agence Nationale de la Recherche (ANR: MERCi, COMMIT, MITHRA), Vaincre la Mucoviscidose, Fondation pour la Recherche Médicale (FRM) [DE2013032644], and Fondation ARC to P.B. and L.K. IBDM authors acknowledge France-BioImaging infrastructure funding ‘Investissements d’Avenir’ [ANR-10-INSB-04-01].


