

# Opposing Fgf and Bmp activities regulate the specification of olfactory sensory and respiratory epithelial cell fates

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## SUMMARY

The olfactory sensory epithelium and the respiratory epithelium are derived from the olfactory placode. However, the molecular mechanisms regulating the differential specification of the sensory and the respiratory epithelium have remained undefined. To address this issue, we first identified *Msx1/2* and *Id3* as markers for respiratory epithelial cells by performing quail chick transplantation studies. Next, we established chick explant and intact chick embryo assays of sensory/respiratory epithelial cell differentiation and analyzed two mice mutants deleted of *Bmpr1a*; *Bmpr1b* or *Fgfr1*; *Fgfr2* in the olfactory placode. In this study, we provide evidence that in both chick and mouse, Bmp signals promote respiratory epithelial character, whereas Fgf signals are required for the generation of sensory epithelial cells. Moreover, olfactory placodal cells can switch between sensory and respiratory epithelial cell fates in response to Fgf and Bmp activity, respectively. Our results provide evidence that Fgf activity suppresses and restricts the ability of Bmp signals to induce respiratory cell fate in the nasal epithelium. In addition, we show that in both chick and mouse the lack of Bmp or Fgf activity results in disturbed placodal invagination; however, the fate of cells in the remaining olfactory epithelium is independent of morphological movements related to invagination. In summary, we present a conserved mechanism in amniotes in which Bmp and Fgf signals act in an opposing manner to regulate the respiratory versus sensory epithelial cell fate decision.

**KEY WORDS:** Bmp, Fgf, Chick, Mouse, Development, Olfactory placode, Respiratory, Sensory

## INTRODUCTION

Early in vertebrate development, the anterior neural plate border gives rise to olfactory placodal cells. Subsequently, the olfactory placode becomes subdivided into the sensory and the respiratory epithelium in the nasal cavity. The sensory epithelium consists of, among other cell types, the olfactory sensory neurons, which transduce odor signals, whereas the respiratory non-neural epithelium removes particles from inhaled air. Although the functions of these mature cell types are well studied, the molecular mechanisms that direct placodal cells into sensory and respiratory epithelial fates remain poorly defined.

At gastrula and head fold stages, prospective olfactory placodal cells are intermingled with lens placodal progenitors at the anterior neural plate border region but, by the neural fold stage, olfactory and lens placodal progenitor cells have become spatially separated (Bhattacharyya et al., 2004; Sjödal et al., 2007; Toro and Varga, 2007). At stage 14 (~22 somites) in chick (Hamburger and Hamilton, 1992) and embryonic day 9.5 (E9.5) in mouse, the olfactory placode becomes visible as an ectodermal thickening, which subsequently invaginates to form the olfactory pit. During this period, the olfactory placode consists of cells of the sensory neuronal lineage at distinct stages of differentiation (Maier and Gunhaga, 2009). In chick, the sensory and the respiratory epithelium can be distinguished morphologically around stage 25. The respiratory epithelium is thinner and located adjacent to the nasal openings,

whereas the sensory epithelium is thicker and lies in a more distal position inside the head mesenchyme (Croucher and Tickle, 1989). The question of how olfactory placodal cells are subdivided into sensory and respiratory epithelial cells was raised 20 years ago (Croucher and Tickle, 1989), but remains unanswered.

Recently, fibroblast growth factor (Fgf) and bone morphogenetic protein (Bmp) signals have been shown to influence the development of the olfactory placode (Sjödal et al., 2007). During late gastrulation, Bmp signals induce olfactory and lens placodal character in anterior neural plate border cells, and sustained exposure of placodal progenitors to Bmp signals promotes the generation of lens cells at the expense of olfactory placodal cells (Sjödal et al., 2007). At this stage, Fgf activity in the neural plate border prevents prospective olfactory/lens placodal cells from acquiring epidermal fate (Sjödal et al., 2007). At later stages of development, Fgf signals promote proliferation and thus increase the number of neural cells within the sensory epithelium (Kawauchi et al., 2004; Kawauchi et al., 2005; LaMantia et al., 2000). The action of Bmps remains less clear: one study has suggested that Bmp signals inhibit neurogenesis (Shou et al., 1999), whereas another argues for a role in neurogenesis within the sensory epithelium (Shou et al., 2000). Whether and how Bmp and/or Fgf signals regulate the differential specification of the sensory epithelium and the respiratory epithelium has not been addressed.

Here we elucidate the roles of Bmp and Fgf activity in the specification of the olfactory sensory and respiratory epithelial cell fate. Our results provide evidence that, in both chick and mouse, Fgf signals are required for the generation of sensory epithelial cells and Bmp activity promotes respiratory epithelial character. Moreover, the lack of Bmp or Fgf activity results in disturbed placodal invagination; however, the fate of cells in the remaining olfactory epithelium differs depending on whether Bmp or Fgf activity is

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inhibited. These findings imply that Fgf and Bmp signals specify sensory and respiratory epithelial cell fates, respectively, independent of olfactory placodal invagination. Finally, our results provide evidence that, during the generation of the sensory and respiratory epithelial domains, Fgf activity suppresses and restricts the ability of BMP signals to induce respiratory cell fate in the nasal epithelium.

## MATERIALS AND METHODS

### Embryos

Fertilized White Leghorn chicken eggs and quail eggs were obtained from Agrisera AB, Umeå, Sweden. Chick embryos were staged according to the protocol of Hamburger and Hamilton (Hamburger and Hamilton, 1992). The use of chick (*Gallus gallus*) embryos in this study was approved by the Ethical Committee on Animal Experiments for Northern Sweden. Floxed alleles of *Fgfr1* and *Fgfr2* or *Bmpr1a* and *Bmpr1b* were used to generate olfactory placodal knockouts when crossed to *Foxg1<sup>Cre</sup>* mice, as previously described (Fernandes et al., 2007; Gutin et al., 2006). The use of mice (*Mus musculus*) was approved by AECOM Animal Institute Committee and Office of Laboratory Animal Welfare.

### Chick quail grafting experiments

Explants of the posterior rim and anterior-medial region of the olfactory pit were isolated from stage 21–23 quail embryos. To facilitate mesenchyme removal, Dispase II (Roche Diagnostics) was used. Explants were briefly incubated in DiI (Cell Tracker CM-DiI, Molecular Probes) to enhance visibility in the egg and homotopically transplanted into stage 21–23 chick embryos using tungsten needles as knives. Embryos were cultured in ovo to approximately stage 27.

### Isolation and culture of tissue explants

Olfactory placodal (OP) explants from stage 14 chick embryos were isolated using tungsten needles and cultured in collagen (Invitrogen) in serum-free OPTI-MEM (Gibco) containing N2 supplement (Invitrogen) and fibronectin (Sigma). To facilitate mesenchyme removal, Dispase II was used. Explants removed from mesenchyme without Dispase II treatment and cultured generated the same combination of cells as Dispase II-treated explants cultured alone (data not shown). Human BMP4 (R&D Systems) was used at 35 ng/ml, recombinant soluble human FGF4 (R&D Systems) was used at 300 ng/ml and recombinant soluble human FGF8b (R&D Systems) was used at 500 ng/ml together with 0.5 µg/ml heparin (Sigma). SU5402 (Calbiochem) was used at 5–10 µM. Soluble noggin and control-conditioned medium were obtained from stably transfected or untransfected Chinese hamster ovary (CHO) cells (Lamb et al., 1993) and cultured in CHO-S-SFM II media (Gibco). noggin-conditioned media was used at an estimated concentration of 50 ng/ml. Explants cultured in the presence of control CM generated the same combination of cells as explants cultured alone (data not shown).

### In ovo electroporation

Stage 12, 17 and 21 chick embryos were electroporated in the olfactory placodal/epithelial region with pCaggs-*GFP* (0.75 µg/µl) (Andersson et al., 2006) alone, or together with pMiwIII-noggin (Timmer et al., 2002), pMiwIII-*Alk6* (James and Schultheiss, 2005), pCaggs-*Fgfr3c-ΔC* (Hasegawa et al., 2004) or pCaggs-*Fgf8b* (Delfini et al., 2005), all at 1.0 µg/µl. DNA was transferred using an Electro Square Porator ECM 830 (BTX Inc.). Stage 12 chick embryos were electroporated by applying five pulses (15 V, 25 ms duration) at 1 second intervals. Stage 17 and 21 chick embryos were electroporated by applying five pulses (20 V, 25 ms duration) at 1 second intervals.

### In situ hybridization and immunohistochemistry

For the use of digoxigenin RNA in situ hybridization and immunohistochemistry, embryos and explants were fixed as described (Sjödahl et al., 2007) and serially sectioned at 8–10 µm. In situ RNA hybridization using chick digoxigenin-labeled *Hes5-1* (Fior and Henrique, 2005), *Ngn1* (Perez et al., 1999), *Id3* (Kee and Bronner-Fraser, 2001) and *Erm* (Hippenmeyer et al., 2002) probes, and mouse digoxigenin-labeled *Hes5* (Machold et al., 2007) and *Id3* (P. Svensson, PhD thesis, Umeå

University, 2008) probes was performed essentially as described (Wilkinson and Nieto, 1993). For radioactive RNA in situ analysis, 10–12 mm sections were hybridized with mouse 35S-labeled *Msx1* (Liu et al., 2004) and *Lhx2* (Rodríguez-Esteban et al., 1998) probes as previously described (Rantze et al., 1994). Antibodies used were as follows: rabbit anti-Sox2 (1:500), rabbit anti-LH2A/B (1:8000) (Lee et al., 1998), rabbit anti-pSmad1/5/8 (Cell Signaling, 1:800), rabbit anti-p-Histone3 (Millipore, 1:500), mouse anti-aCaspase3 (Cell Signaling, 1:1000), mouse anti-HuC/D (Molecular Probes, 1:200), mouse anti-*Msx1/2* (4G1, Developmental Studies Hybridoma Bank, 1:20), mouse anti-Tuj1 (Covance, 1:500) and mouse anti-QPCN – a quail-specific antibody (Developmental Studies Hybridoma Bank, 1:200). Nuclei were stained using DAPI (Sigma).

### Statistical analyses

To determine the percentage of HuC/D-, *Msx1/2*-, active caspase 3 (aCaspase3)- and phosphorylated histone 3 (pHistone3)-expressing cells per explant and in electroporated GFP-positive regions in vivo, the number of antigen-expressing cells was quantified and compared with the total number of cells, determined by DAPI-stained nuclei. The graphs represent the mean number of cells positively stained for HuC/D and *Msx1/2*, respectively. Error bars represent ± 1 s.e.m. *P*-values were obtained comparing controls with the treated explants, and comparing GFP-control with GFP-construct-electroporated embryos using a Student's *t*-test.

### Western blot analysis

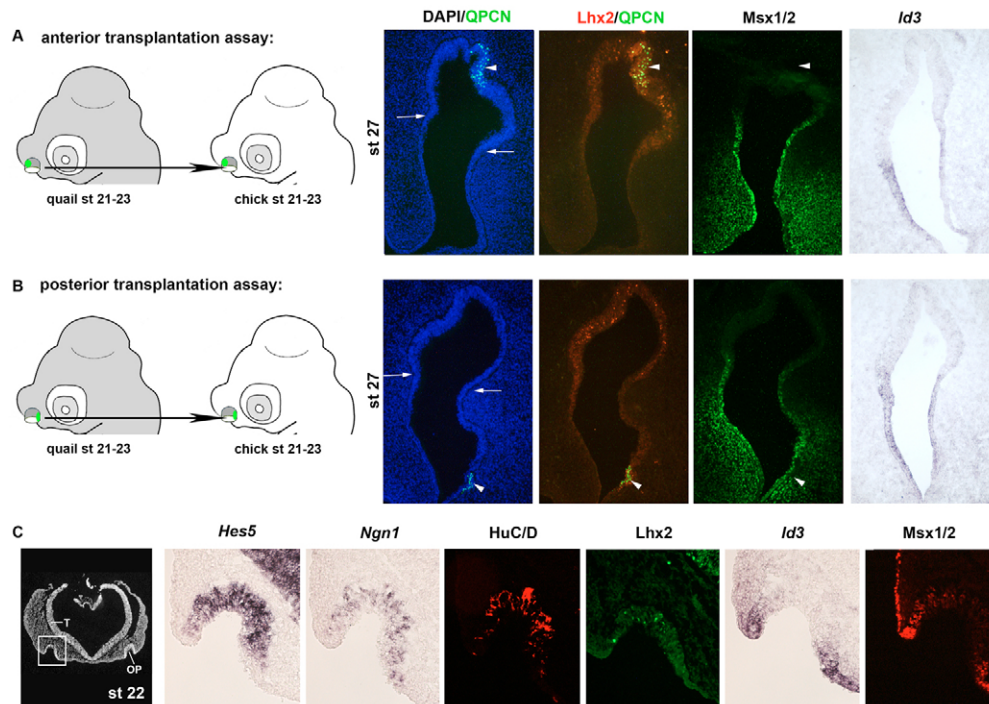
Stage 14 OP explants (*n*=20) were isolated and cultured for 1.5 hours on a Millicell membrane (PICM01250, Millipore) in serum-free OPTI-MEM (Gibco-BRL). The explants were homogenized by 10-minute incubation in lysis buffer at 100°C. Tissue extracts containing 3.5 µg total protein were applied to each well. Antibodies used were rabbit anti-phosphorylated Smad1/5/8 (Cell Signaling, 1:5000), anti-diphosphorylated Erk (M9692, Sigma) and anti-β-actin (Cell Signaling, 1:10,000).

## RESULTS

### Prospective respiratory epithelial cells express *Msx1/2* and *Id3*

In chick embryos, the specification of olfactory placodal cells is initiated at the late gastrula stage (Sjödahl et al., 2007), but when and how cells in the olfactory placode subsequently are subdivided into sensory and respiratory epithelial cells remains unclear. In this study, sensory epithelial cells at different maturation levels in the sensory neuronal lineage are detected by the expression of the following: *Hes5-1* (Fior and Henrique, 2005), hereafter referred to as *Hes5*, which defines progenitor cells (Cau et al., 2000); *Ngn1*, which marks immediate neuronal precursors (Cau et al., 2002); members of the Hu class of RNA-binding proteins (HuC/D), which mark postmitotic neurons (Fornaro et al., 2003); and the transcription factor *Lhx2*, which identifies olfactory sensory neurons (Hirota and Mombaerts, 2004; Kolterud et al., 2004).

Because well-described markers defining respiratory epithelial cells were unavailable, we first determined the location of prospective respiratory epithelial cells by performing quail–chick grafting experiments. Small cell populations either from the anteromedial region or the posterior rim of the invaginating olfactory pit of stage 21–23 quail embryos were dissected out and grafted into stage 21–23 host chick embryos at a homotopic position (Fig. 1A,B). Chick embryos were allowed to develop to stage 27 and the position of the grafted quail cells was determined by using a quail-specific antibody (Fig. 1A,B). The quail-derived anteromedial cells were observed in the sensory epithelium in stage 27 chick embryos (7/7; Fig. 1A), which is consistent with previous findings that sensory epithelial cells are located primarily in the anteromedial region of the olfactory pit at stage 22 (Maier and Gunhaga, 2009). By contrast, the quail-derived posterior rim cells were observed in



**Fig. 1. Prospective respiratory epithelial cells express *Id3* and *Msx1/2*.** In ovo quail–chick grafts performed at stage 21–23 and analyzed at ~stage 27. (A,B) Schematic drawing indicating the position of the homotopic anteromedial graft (A,  $n=7/7$ ) and posterior rim graft (B,  $n=8/8$ ). The grafted quail tissue is detected by the expression of QPCN (green cells, arrowheads). Arrows indicate the border between the thicker sensory and the thinner respiratory epithelium. Anteromedial grafts are detected in the sensory epithelium in an *Lhx2*<sup>+</sup> region, whereas posterior rim grafts are detected in the thinner respiratory epithelium in an *Msx1/2*<sup>+</sup> and *Id3*<sup>+</sup> region of the olfactory pit. (C) To the left, DAPI-stained transversal section of the anterior head at stage 22. The boxed area indicates the prospective olfactory pit (OP) adjacent to the telencephalon (T). At stage 22, cells in the anteromedial part of the olfactory pit express *Hes5*, *Ngn1*, *HuC/D* and *Lhx2*, and cells at the rim of the olfactory pit express *Id3* and *Msx1/2*. *Hes5* and *Lhx2* can also be detected in the telencephalon.

the respiratory epithelium (8/8; Fig. 1B), providing evidence that precursor cells of the respiratory epithelium are located at the posterior rim of the olfactory pit at stage 21–23.

We next analyzed the expression pattern of putative markers defining respiratory cells. At stage 22–27, the helix-loop-helix gene *Id3* and the transcription factor *Msx1/2* are expressed in the rim of the olfactory pit, corresponding to the rim-grafted region (Fig. 1A–C). By contrast, cells of the sensory neuronal lineage detected by *Hes5*, *Ngn1*, *HuC/D* and *Lhx2* expression are located in the anteromedial part of the olfactory pit in a pattern distinct from that of *Id3*<sup>+</sup> and *Msx1/2*<sup>+</sup> cells (Maier and Gunhaga, 2009). Taken together, prospective respiratory epithelial cells are detected at the rim and in the posterior part of the olfactory pit and can be identified by the expression of *Id3* and *Msx1/2*.

### Early specification of olfactory placodal cells as sensory and respiratory epithelial character

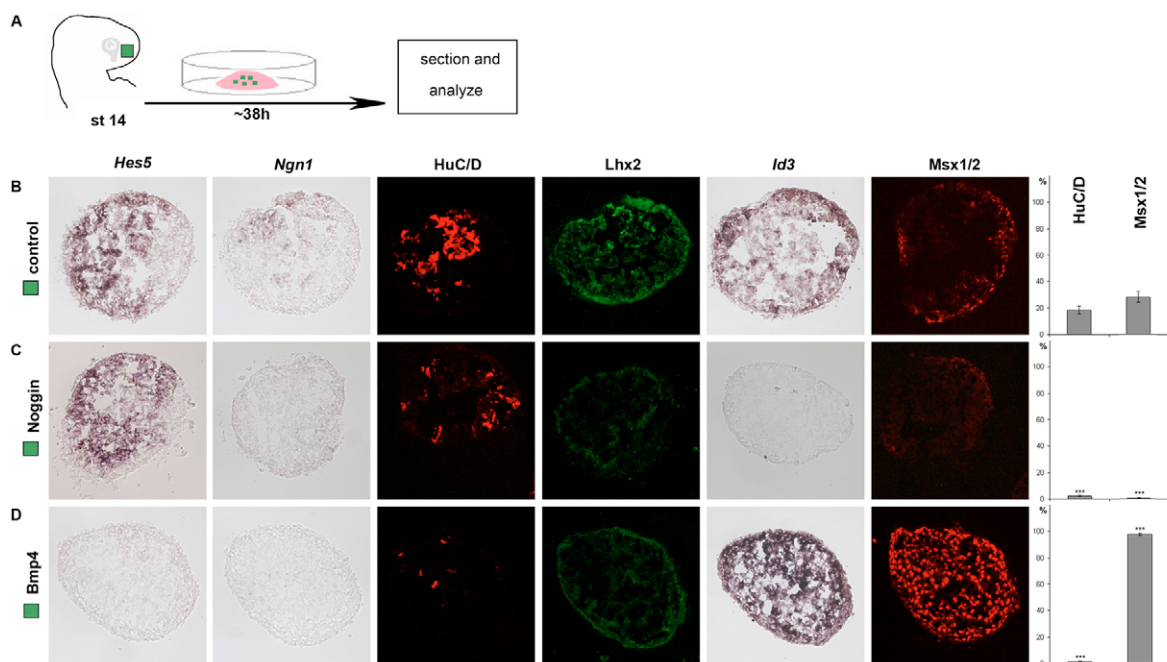
To examine the mechanism by which sensory and respiratory epithelial cells are specified, we established an explant assay of sensory/respiratory epithelial cell differentiation by culturing olfactory placodal (OP) explants of stage 14 chick embryos (Fig. 2A). OP explants were cultured for ~38 hours, corresponding in time to approximately stage 22 embryos, a stage when sensory and respiratory epithelial domains can be distinguished by molecular markers (Fig. 1C). The underlying head mesenchyme was removed to avoid an indirect influence of these cells. OP explants generated *Hes5*<sup>+</sup>, *Ngn1*<sup>+</sup>, *HuC/D*<sup>+</sup> and *Lhx2*<sup>+</sup> cells, characteristic of the sensory epithelium, as well as *Id3*<sup>+</sup> and *Msx1/2*<sup>+</sup> cells, characteristic of the

respiratory epithelium, in distinct regions of the explants (Fig. 2B). Approximately 25% of the cells in the explants were *Msx1/2*<sup>+</sup> (Fig. 2B; see Fig. S1 in the supplementary material), indicative of respiratory epithelial character, whereas approximately 75% of the cells expressed *HuC/D* or the neural precursor marker *Sox2* (see Fig. S1A,B in the supplementary material), characteristic of the sensory epithelial region at stage 22 (see Fig. S1C,D in the supplementary material). Thus, already at stage 14, when the olfactory placode is first morphologically visible, cells in the olfactory placode are specified as sensory and respiratory epithelial cells.

### Bmp signals promote the generation of respiratory epithelial cells

*Bmp* activity has been shown to induce olfactory placodal cells (Sjödahl et al., 2007), prompting us to examine whether *Bmp* signals also regulate the subdivision of sensory and respiratory epithelial cell fate. We first examined the pattern of expression of *Bmp4* and its downstream signaling mediator phosphorylated (p) *Smad1/5/8*, in the olfactory placode and pit region of stage 14 and 22 chick embryos. At stage 14, *Bmp4* was expressed preferentially in the posterior part of the olfactory placode (see Fig. S2A,B in the supplementary material). At stage 22, *Bmp4* was expressed at the rim in the posterior half of the olfactory pit (see Fig. S2C–E in the supplementary material) and p*Smad1/5/8* was expressed in almost every cell in the posterior part, as well as in scattered cells in the anteromedial part of the olfactory pit (see Fig. S2F–H in the supplementary material). Next, we addressed whether *Bmp* signals are required for the generation of respiratory and/or sensory





**Fig. 2. Bmp signals promote the generation of cells of respiratory epithelial character.** (A) Schematic drawing depicting the explant method. Stage 14 olfactory placodal (OP) explants (green box) were isolated, separated from the head mesenchyme and cultured *in vitro* to the developmental equivalent time-point of stage 22. (B) OP explants ( $n=30$ ) cultured alone generated *Hes5*<sup>+</sup>, *Ngn1*<sup>+</sup>, HuC/D<sup>+</sup> and *Lhx2*<sup>+</sup> sensory epithelial cells, and *Id3*<sup>+</sup> and *Msx1/2*<sup>+</sup> respiratory epithelial cells. (C) In OP explants cultured together with noggin ( $n=18$ ), the generation of *Ngn1*<sup>+</sup>, *Lhx2*<sup>+</sup>, *Id3*<sup>+</sup> and *Msx1/2*<sup>+</sup> was blocked, HuC/D<sup>+</sup> neurons reduced, and most cells expressed *Hes5*. (D) In OP explants ( $n=18$ ), Bmp4 (35 ng/ml) blocked the generation of *Hes5*<sup>+</sup>, *Ngn1*<sup>+</sup> and *Lhx2*<sup>+</sup> sensory epithelial cells, reduced the numbers of HuC/D<sup>+</sup> neurons and induced *Id3*<sup>+</sup> and *Msx1/2*<sup>+</sup> respiratory epithelial cells. Bars represent the mean number of cells positively stained for HuC/D and *Msx1/2*, respectively. Error bars represent mean  $\pm$  1 s.e.m. \*\*\*,  $P < 0.001$ .

epithelial character by culturing OP explants together with noggin, a known Bmp inhibitor (Lamb et al., 1993). In the presence of noggin, most cells expressed *Hes5*, characteristic of progenitor cells in the sensory epithelium, whereas the generation of *Id3*<sup>+</sup> and *Msx1/2*<sup>+</sup> respiratory epithelial cells, *Ngn1*<sup>+</sup> neuronal precursors, and HuC/D<sup>+</sup> and *Lhx2*<sup>+</sup> sensory neurons was strongly downregulated or completely abolished (Fig. 2C). Thus, at stage 14, Bmp signals are required for the generation of respiratory epithelial cells and sensory neurons and, in the absence of Bmp activity, olfactory placodal cells acquire sensory epithelial progenitor character.

To analyze whether, at stage 14, elevated levels of Bmp signals promote the generation of respiratory epithelial cells or sensory neurons, we exposed OP explants to Bmp4 (35 ng/ml). Under these conditions, the generation of *Hes5*<sup>+</sup>, *Ngn1*<sup>+</sup> and *Lhx2*<sup>+</sup> cells was blocked, and the numbers of HuC/D<sup>+</sup> neurons were markedly reduced, whereas *Id3*<sup>+</sup> and *Msx1/2*<sup>+</sup> cells, indicative of respiratory epithelial character, were increased (Fig. 2D). Thus, additional Bmp signals in olfactory placodal cells induce respiratory epithelial character at the expense of sensory epithelial cells. Taken together, these results provide evidence that, at olfactory placodal stages, Bmp activity is both required and sufficient to specify respiratory epithelial cells.

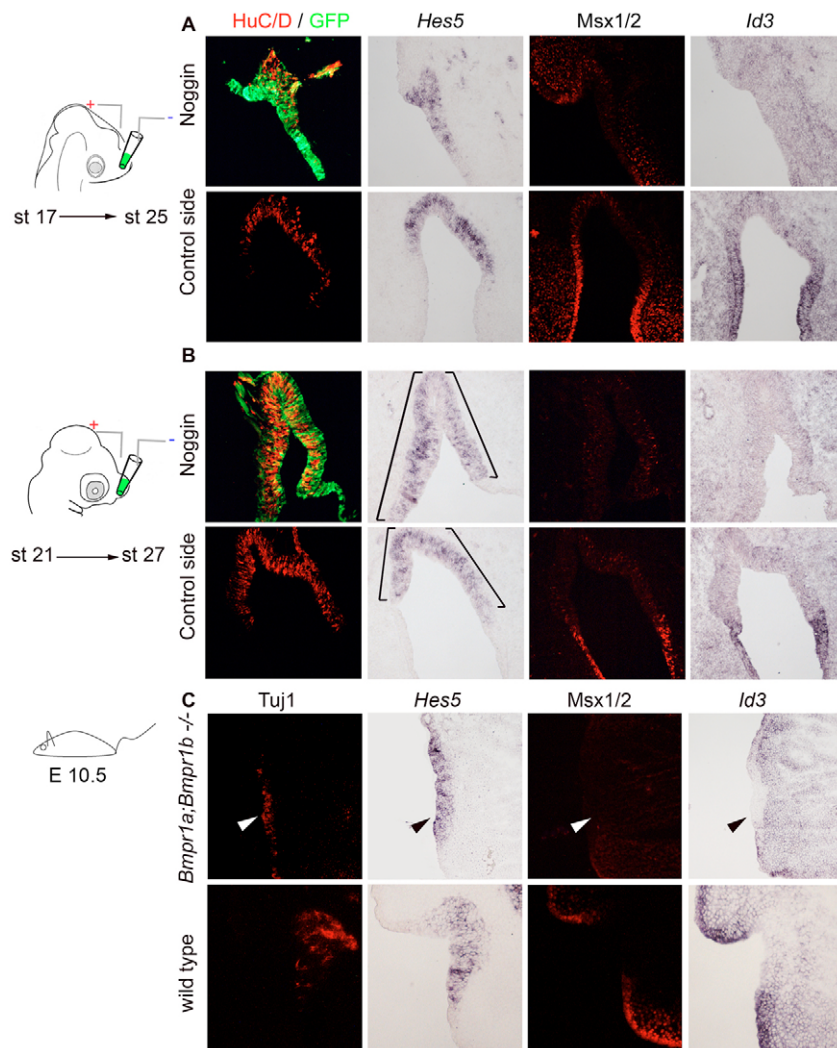
### Bmp activity is required for the specification of respiratory epithelial cells in intact chick and mouse embryos

Next we examined the temporal requirement of Bmp activity for the specification of respiratory epithelial cells *in vivo*. To address this question, stage 12, 17 and 21 chick embryos were electroporated in

ovo in the olfactory placode or pit region to transfer a control green fluorescent protein (GFP) vector, alone or together with a noggin vector (Timmer et al., 2002), and cultured for ~48 hours to approximately stage 22, 25 and 27, respectively. Embryos with GFP staining within the olfactory region were selected for further analyses.

All control-electroporated embryos (8/8) exhibited normal morphology of the olfactory pit and a normal expression pattern of *Hes5*; HuC/D and *Id3*; *Msx1/2*, defining sensory and respiratory epithelial cells, respectively (see Fig. S3A in the supplementary material). By contrast, on the noggin-transfected side, expression of *Id3* and *Msx1/2* was reduced or abolished, whereas *Hes5*<sup>+</sup> and HuC/D<sup>+</sup> cells were generated in the remaining nasal epithelium (22/22; Fig. 3A,B; see Fig. S3B in the supplementary material). Thus, ongoing Bmp activity is required for the specification of respiratory epithelial cells. In addition, noggin-transfected embryos exhibited a less-invaginated olfactory pit (22/22; Fig. 3A,B; see Fig. S3B in the supplementary material). At stages 25 and 27, no respiratory epithelial cells were detected and an expanded sensory epithelial domain spanned the entire compressed olfactory pit (15/15; Fig. 3A,B).

To determine whether a change in proliferation and/or cell death accounted for the disrupted invagination, we analyzed the expression of the cell proliferation marker phosphorylated (p) histone 3 and cell death marker activated (a) caspase 3. In noggin-electroporated embryos, there was a decrease in cell proliferation but no change in cell death (see Fig. S4B in the supplementary material) compared with control GFP-electroporated embryos (see Fig. S4A in the supplementary material). Thus, in the absence of Bmp activity, decreased proliferation within the olfactory placode appears to disrupt epithelial invagination.



**Fig. 3. Inhibition of Bmp signals results in loss of respiratory epithelium in vivo.** (A,B) In ovo electroporation of stage 17 (A,  $n=7$ ) and 21 (B,  $n=8$ ) embryos in the olfactory placodal region using pCaggs-*GFP* together with pMiwIII-noggin and cultured to stage 25 and 27, respectively. noggin-electroporation resulted in disturbed invagination of the nasal epithelium and reduced, or loss of, *Id3* and *Msx1/2* expression and remained or increased *Hes5* and *HuC/D* expression throughout the nasal epithelium. Brackets in B indicate the expanded sensory epithelium in noggin-electroporated embryos. (C) The invagination of the olfactory placode in E10.5 *Foxg1:Bmpr1a;Bmpr1b* mutant mice ( $n=4$ ) was disturbed, the expression of *Msx1/2* and *Id3* was greatly reduced or completely lost, and *Hes5* and *Tuj1* expression was detected throughout the presumptive olfactory placodal region (arrowheads). E10.5 wild-type mice expressed *Hes5* and *Tuj1* in the anteromedial region and *Msx1/2* and *Id3* at the rim of the olfactory pit.

To further evaluate the requirement of Bmp activity for the specification of respiratory epithelial cells in vivo, we analyzed *Bmpr1a;Bmpr1b* double-mutant mice, in which *Bmpr1a* and *Bmpr1b* have been deleted in the olfactory placode by using the *Cre/loxP* system under the control of the *Foxg1* promoter (Fernandes et al., 2007). *Foxg1* expression is first detected around E8.0 in mouse (~stage 8 in chick) in the anterior neural ridge close to the anterior neural folds where prospective olfactory placodal cells reside and, at later stages, *Foxg1* is expressed in the olfactory placode (Bailey et al., 2006; Hebert and McConnell, 2000; Shimamura et al., 1995).

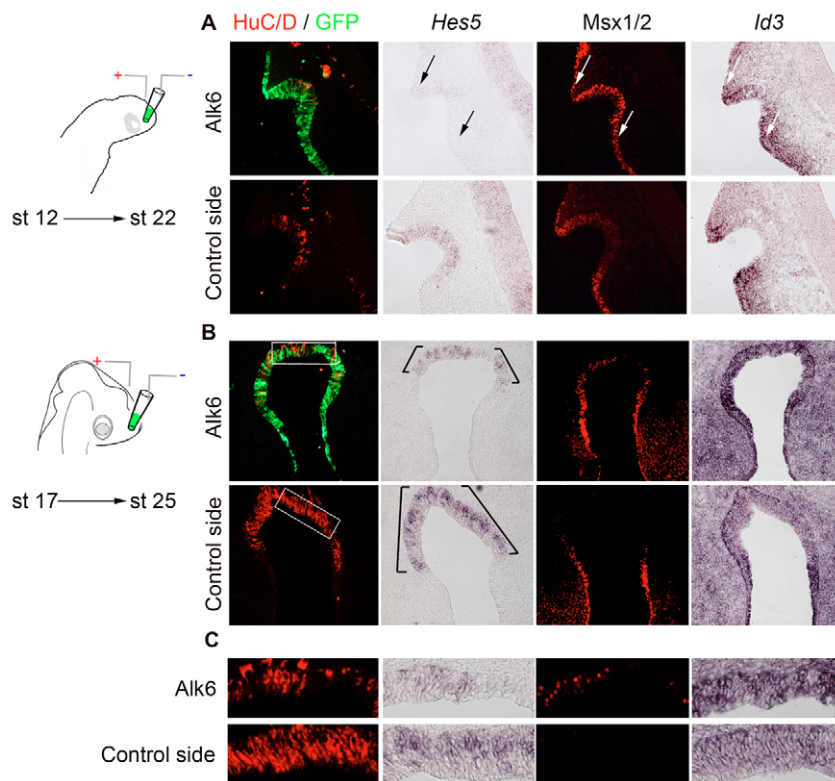
In wild-type E10.5 mice, the expression pattern of *Hes5* and sensory neurons, here detected by *Tuj1* (Lee et al., 1990) and *Lhx2*, in the medial part of the olfactory placode and *Id3* and *Msx1/2* in the rim of the olfactory placode (Fig. 3C; see Fig. S5 in the supplementary material) resembled the expression pattern detected in the chick olfactory placode (Fig. 1C). In E10.5 *Bmpr1a;Bmpr1b* double mutants, the prospective olfactory placodal ectoderm was thickened but failed to invaginate properly, resulting in an absent or severely reduced nasal cavity (4/4; Fig. 3C; see Fig. S5 in the supplementary material). Consistent with our chick data, in Bmp-inhibited mouse mutants examined by aCaspase3 and pHistone3 markers, there was no change in cell death but cell proliferation appeared to be decreased compared with wild-type embryos (see

Fig. S6A,B in the supplementary material). Moreover, the loss of *Bmpr1a* and *Bmpr1b* in the olfactory placode resulted in strongly reduced or complete loss of *Id3* and *Msx1/2* expression, whereas the expression of *Hes5*, *Tuj1* and *Lhx2* extended through most of the compressed olfactory placode (Fig. 3C; see Fig. S5 in the supplementary material). This phenotype demonstrates that Bmp signals are also required to generate respiratory epithelial cells in the mouse olfactory placode. Moreover, our data from both mouse and chick suggest that Bmp signals are not essential for neurogenesis in the sensory epithelium. Collectively, these results suggest a conserved mechanism in amniotes in which Bmp signals are required for the generation of respiratory epithelial cells and, in the absence of Bmp activity, cells within the olfactory placode and pit acquire sensory epithelial character.

### Elevated levels of Bmp activity promotes the generation of respiratory epithelial cells at the expense of sensory epithelial cells in intact chick embryos

Next, we analyzed how elevated levels of Bmp activity affect the subdivision of respiratory and sensory epithelial cells in the nasal epithelium in vivo. Stage 12, 17 and 21 chick embryos were electroporated in ovo in the olfactory placode or pit region to transfer a constitutively active Bmp receptor, *Alk6* (*Bmpr1b*), vector (James





**Fig. 4. Bmp activity specifies the respiratory epithelium in vivo.** (A) In ovo electroporation of stage 12 embryos in the OP region using pMiwIII-*Alk6* ( $n=9$ ) resulted in upregulated expression of *Id3* and *Msx1/2*, downregulation of *Hes5* expression (arrows) and a moderate decrease in *HuC/D* neurons, when analyzed at stage 22. *HuC/D* and *Hes5* can also be detected in the telencephalon. (B) In ovo *Alk6*-electroporation of stage 17 embryos ( $n=6$ ) resulted in upregulation of *Id3* and *Msx1/2* expression throughout the entire extent of the nasal epithelium, whereas *Hes5* and *HuC/D* expression was restricted to a proportionally smaller area (brackets) when analyzed at stage 25. (C) Higher magnification figures of the sensory epithelium region, as indicated by white boxes in B.

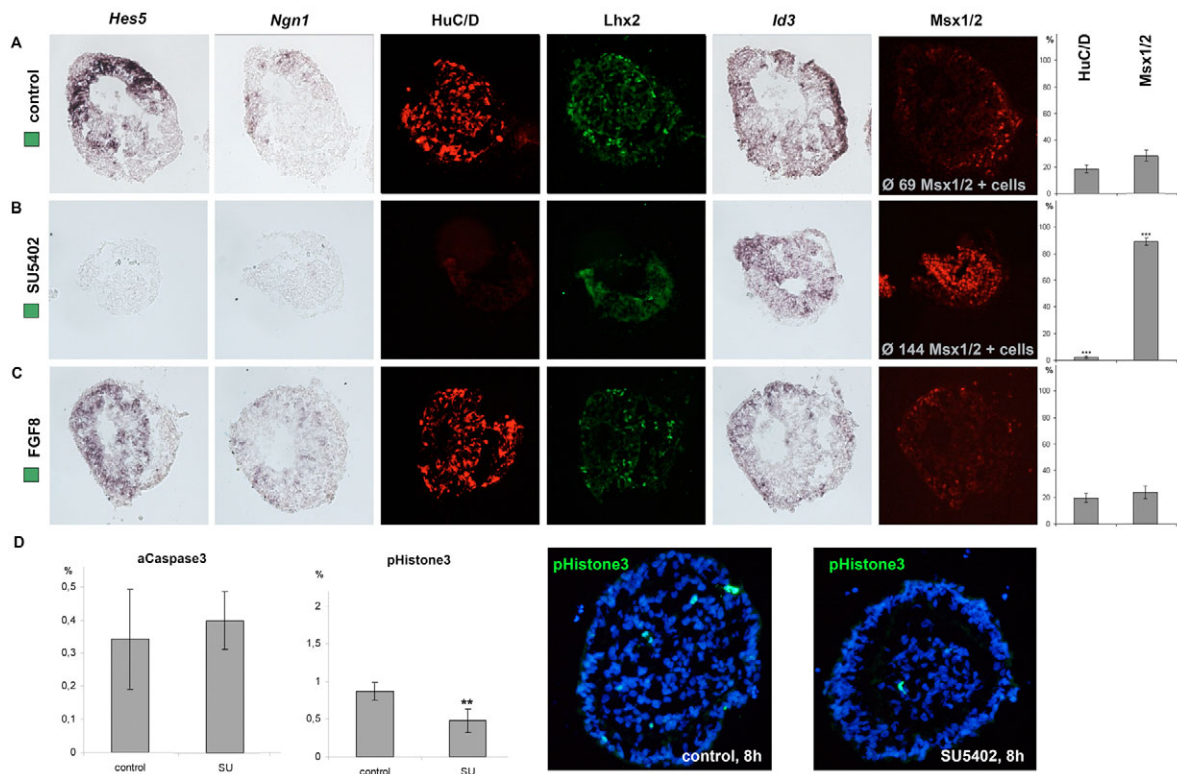
and Schultheiss, 2005), together with a control GFP vector, and were cultured to approximately stages 22, 25 and 27, respectively. The *Alk6*-transfected stage 12 embryos exhibited normal olfactory placode morphology (9/9; Fig. 4A), whereas the medial epithelium in the olfactory pit of stage 17 electroporated embryos appeared to be thinner compared with the control side (6/6; Fig. 4B,C). However, no change in pHistone3 or aCaspase3 expression was detected in stage 17 *Alk6*-transfected embryos compared with control embryos (see Fig. S4D,E in the supplementary material). At both stages, activation of the Bmp pathway resulted in a substantial upregulation of *Id3* and *Msx1/2* expression, a reduction of *Hes5*-expressing cells and a decrease in *HuC/D* neurons in the electroporated domain (13/15; Fig. 4A-C). By contrast, in stage 21 *Alk6*-transfected embryos (5/5), *Id3* and *Msx1/2* expression was only induced in a few scattered cells in the sensory epithelium, and *Hes5* and *HuC/D* expression remained mostly unaffected (see Fig. S3C in the supplementary material). Collectively, these results provide evidence that ongoing Bmp activity is required for the specification of respiratory epithelial cells and that, prior to stage 21, elevated Bmp signals induce respiratory epithelial character in prospective sensory epithelial cells.

### Fgf signaling is required for the generation of sensory epithelial cells in vitro

We next turned to the issue of how sensory epithelial cells are specified. As fibroblast growth factor (Fgf) signals have been shown to affect the early development of the olfactory placode (Sjödahl et al., 2007), we first examined the expression pattern of *Fgf8* and the Fgf downstream mediator *Erm* in the olfactory placode and pit region of stage 14 and 22 chick embryos. At both stages 14 and 22, *Fgf8* and *Erm* were expressed preferentially in the anterior part of the olfactory placode and pit (see Fig. S2A-H in the supplementary material). Second, we analyzed whether Fgf signals play a role in

the differential specification of sensory and respiratory epithelial cells. To assess this, we first cultured stage 14 OP explants in the presence of SU5402 (5–10  $\mu$ M), an inhibitor of Fgf receptor signaling (Mohammadi et al., 1997). Under these conditions, the generation of *Hes5*<sup>+</sup>, *Ngn1*<sup>+</sup>, *HuC/D*<sup>+</sup> and *Lhx2*<sup>+</sup> sensory epithelial cells was suppressed, and most cells expressed *Msx1/2* and *Id3*, characteristic of respiratory epithelial cells (Fig. 5B). The smaller size of the SU5402-treated explants was due to a decrease in cell proliferation, as analyzed by pHistone3 expression, without any change in cell death, as examined by aCaspase3 expression (Fig. 5D). Although cell proliferation was decreased in SU5402-treated explants compared with control explants, the total amount of *Msx1/2*<sup>+</sup> cells was increased in Fgf-inhibited explants (Fig. 5A,B), indicating that inhibition of Fgf activity leads to a cell fate switch. Thus, Fgf signals are required for the generation of sensory epithelial cells and, in the absence of Fgf activity, olfactory placodal cells acquire respiratory epithelial character.

We next examined whether levels of Fgf activity affect the differential generation of sensory and respiratory epithelial cells in olfactory placodal cells, by culturing OP explants in the presence of Fgf4 (300 ng/ml), or Fgf8 (500 ng/ml). Under these conditions, the generation of *Hes5*<sup>+</sup>, *Ngn1*<sup>+</sup>, *HuC/D*<sup>+</sup> and *Lhx2*<sup>+</sup> sensory epithelial cells resembled the generation of cells in control explants, and the number of *Msx1/2*<sup>+</sup> and *Id3*<sup>+</sup> respiratory epithelial cells was not significantly decreased (Fig. 5C; data not shown). Moreover, OP explants cultured in the presence of noggin and additional Fgf4 (300 ng/ml) generated the same combination of cell types as explants exposed to noggin only (see Fig. S7A-C in the supplementary material), indicating that additional Fgf exposure does not affect the differential generation of sensory and respiratory epithelial cells. Thus, Fgf activity is required for the specification of sensory epithelial cells but elevated levels of Fgf are not sufficient to induce sensory epithelial cells.



**Fig. 5. Fgf signals are required for the generation of cells of sensory epithelial character.** (A) OP explants ( $n=30$ ) cultured alone generated  $Hes5^+$ ,  $Ngn1^+$ ,  $HuC/D^+$  and  $Lhx2^+$  sensory epithelial cells, and  $Id3^+$  and  $Msx1/2^+$  respiratory epithelial cells. (B) In OP explants cultured together with SU5402 (7.5 nM;  $n=12$ ), the generation of sensory epithelial cells was blocked, and  $Id3^+$  and  $Msx1/2^+$  respiratory epithelial cells were induced. (C) OP explants exposed to Fgf8 (500 ng/ml;  $n=15$ ) generated  $Hes5^+$ ,  $Ngn1^+$ ,  $HuC/D^+$  and  $Lhx2^+$  sensory epithelial cells, and  $Id3^+$  and  $Msx1/2^+$  respiratory epithelial cells. (D) aCaspase3 and pHistone3 analysis in SU5402-treated explants compared with control explants cultured for 8 hours. Bars represent mean number of cells positively stained for HuC/D, Msx1/2, aCaspase3 and pHistone3. Error bars represent mean  $\pm$  1 s.e.m. \*\*\*,  $P<0.001$ ; \*\*,  $P=0.03$ .

### Suppressed Fgf activity in intact chick and mouse olfactory placodes inhibits the specification of sensory epithelial cells

In order to examine the role of Fgf signaling in the olfactory placode in vivo, the olfactory placodal region of stage 12 chick embryos was electroporated in ovo using the GFP vector together with a pCaggs-*Fgf8b* vector (Delfini et al., 2005), or a truncated form of Fgf receptor 3 (pCaggs-*Fgfr3c-ΔC*), which acts as an inhibitor of the Fgf signaling pathway (Hasegawa et al., 2004). Embryos were cultured to approximately stage 22, and embryos with GFP staining within the olfactory region were selected for further analyses.

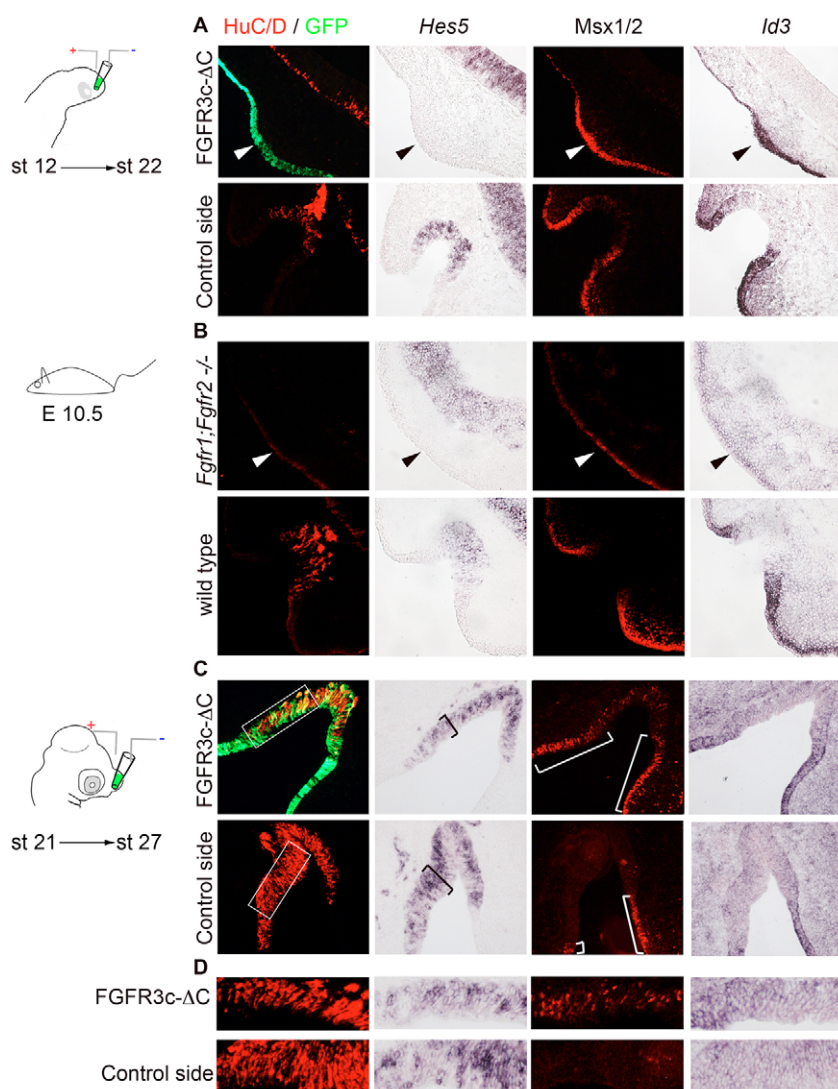
Consistent with our in vitro findings (Fig. 5C), activation of the Fgf pathway did not appear to affect *Hes5*, *HuC/D*, *Msx1/2* or *Id3* expression (22/22; see Fig. S3D in the supplementary material). By contrast, inhibition of Fgf activity reduced or completely blocked expression of *Hes5* and *HuC/D*, and an increase of *Msx1/2* and *Id3* expression in the sensory epithelium was detected, compared with the non-electroporated control side (13/16; Fig. 6A). In the most severely affected embryos, the invagination of the olfactory placode, forming the nasal cavity, was completely missing (4/16; Fig. 6A). The severity of the phenotype was clearly linked to the efficiency of the electroporation, as the amount of transferred GFP and *Fgfr3c-ΔC* correlated with the reduction in levels of *Hes5* and *HuC/D* expression (data not shown). By analyzing pHistone3 and aCaspase3 expression, the disrupted placodal invagination in *Fgfr3c-ΔC*-electroporated embryos appeared to be controlled by

reduced cell proliferation and not by increased cell death compared with control embryos (see Fig. S4A,C in the supplementary material). Thus, our results show that Fgf activity is required for proper invagination of the olfactory placode and indicate that Fgf signals are required for the specification of sensory epithelial cells.

We next analyzed *Fgfr1*;*Fgfr2* double-mutant mice, in which *Fgfr1* and *Fgfr2* have been deleted in the olfactory placode (Gutin et al., 2006). Consistent with our findings in chick, in E10.5 *Fgfr1*;*Fgfr2* double mutants, the ectoderm of the prospective olfactory placodal region failed to thicken and invaginate and the nasal cavity did not form (4/4; Fig. 6B). This phenotype appeared to be caused by a decrease in cell proliferation and not a response to increased cell death as examined by pHistone3 and aCaspase3 markers, respectively (see Fig. S6A,C in the supplementary material). Moreover, the loss of *Fgfr1* and *Fgfr2* in the olfactory placode resulted in a greatly reduced or complete loss of *Hes5* and *HuC/D* expression, whereas *Msx1/2* and *Id3* expression extended through most of the presumptive olfactory placodal region (Fig. 6B). Thus, this phenotype demonstrates that, in mouse, Fgf signaling is required for proper invagination of the olfactory placode and for the development of sensory epithelial cells.

The finding that suppressed Fgf activity disrupts invagination of the olfactory placode raised the question of whether lack of sensory epithelial cells is a secondary effect of disturbed placodal invagination rather than a specific requirement of ongoing Fgf activity. To examine this, we transfected the *Fgfr3c-ΔC* construct





**Fig. 6. Fgf signals are required for the generation of sensory epithelial cells in vivo.**

(A) In ovo electroporation of stage 12 embryos in the olfactory placodal region using pCaggs-*GFP* together with pCaggs-*Fgfr3c-ΔC* ( $n=16$ ) resulted in loss of *Hes5* and *HuC/D* expression and an increase of *Msx1/2* and *Id3* expression in the nasal epithelial region, when analyzed at stage 22. In the most severely affected embryos, the olfactory epithelial region fails to invaginate (arrowheads). *HuC/D* and *Hes5* can also be detected in the telencephalon. (B) In E10.5 *Foxg1:Fgfr1;Fgfr2* mutant mice ( $n=4$ ) the placodal invagination was absent, the expression of *Hes5* and *HuC/D* was greatly reduced or completely lost, and *Msx1/2* and *Id3* expression were detected throughout the presumptive olfactory placodal region (arrowheads). E10.5 wild-type mice expressed *Hes5* and *HuC/D* in the anteromedial region and *Msx1/2* and *Id3* at the rim of the olfactory pit. *Hes5* can also be detected in the telencephalon. (C) In ovo *Fgfr3c-ΔC*-electroporation of stage 21 ( $n=6$ ) embryos resulted in an increase in *Msx1/2*<sup>+</sup> and *Id3*-positive cells in the sensory epithelial domain when analyzed at stage 27. The sensory epithelium, identified by expression of *Hes5* and *HuC/D*, was reduced in size and appeared thinner (black brackets), while the respiratory epithelium was expanded (white brackets). (D) Higher magnification figures of the sensory epithelial region, as indicated by white boxes in C.

into the olfactory pit in stage 17 and 21 chick embryos and cultured them in ovo to approximately stages 25 and 27, respectively. In these embryos, the placodal invagination was not affected. However, the thickness of the sensory epithelium located in the medial part of the pit was reduced on the *Fgfr3c-ΔC*-transfected side (12/12; Fig. 6C, black brackets; data not shown). Moreover, the respiratory epithelial domain was expanded at the expense of the sensory epithelium (12/12; Fig. 6C, white brackets; data not shown). In addition, the generation of *Hes5*<sup>+</sup> cells and *HuC/D*<sup>+</sup> neurons was reduced, and *Id3* and *Msx1/2* expression was upregulated in a scattered pattern in the medial part of the epithelium (Fig. 6C,D; data not shown). Thus, in the normal invaginated olfactory pit, Fgf signaling is required for the generation of sensory epithelial cells. Collectively, these results suggest that Fgf signals are required for the generation of sensory epithelial cells and, in the absence of FGF activity, prospective sensory epithelial cells acquire a respiratory epithelial character.

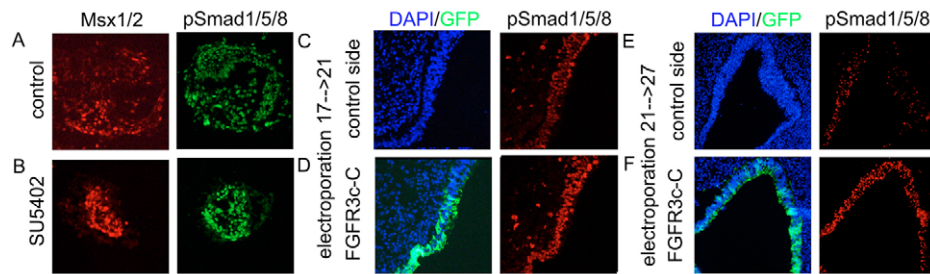
### Fgf signals restrict Bmp activity in the olfactory placode

Our data show that olfactory placodal cells acquire respiratory epithelial character both through inhibition of Fgf signals or through elevated Bmp activity. Therefore, we wanted to test whether the specification of respiratory epithelial character is dependent on Bmp

signaling in the absence of Fgf activity. Stage 14 OP explants cultured in the presence of SU5402 (5 μM) together with noggin generated *Hes5*<sup>+</sup> and *HuC/D*<sup>+</sup> sensory epithelial cells, but no *Msx1/2*<sup>+</sup> or *Id3*<sup>+</sup> respiratory epithelial cells (see Fig. S7E in the supplementary material). Thus, even in the absence of Fgf activity, the specification of respiratory epithelial cells requires intrinsic Bmp signaling. These results provide evidence that Fgf signals act to restrict Bmp activity in the olfactory placode.

To further investigate the potential mechanism of Bmp and Fgf signaling interactions, we monitored the levels of the downstream signaling mediators pSmad1/5/8 and diphosphorylated (dp) ERK, indicative of Fgf signaling via the ERK pathway, by western blotting. Both pSmad1/5/8 and dpERK were detected in OP explants cultured alone for 1.5 hours. As expected, the levels of pSmad1/5/8 were blocked or increased in the presence of noggin or Bmp4 (10ng/ml), respectively (see Fig. S7F in the supplementary material). Moreover, in the presence of SU5402 (10 μM), dpERK was blocked but, at this time-point, no effect on pSmad1/5/8 was detected (see Fig. S7F in the supplementary material). To examine whether inhibition of Fgf signals affected Bmp activity at later time points, we cultured stage 14 OP explants in the presence or absence of SU5402 (5-10 μM) for 24 hours and monitored pSmad1/5/8 expression levels. Stage 14 OP





**Fig. 7. Inhibition of Fgf signals upregulates pSmad1/5/8 in olfactory placodal and sensory epithelial cells.** (A) Cells in OP explants ( $n=6$ ) express low levels of pSmad1/5/8 in the  $Msx1/2^-$  region and higher levels of pSmad1/5/8 in the  $Msx1/2^+$  domain. (B) In the presence of SU5402, cells in OP explants ( $n=6$ ) co-express high levels of pSmad1/5/8 and  $Msx1/2$ . (C–F) In ovo *Fgfr3c-ΔC*-electroporation of stage 17 cultured to stage 21 (C,D) and stage 21 cultured to stage 27 (E,F) embryos ( $n=7$ ) resulted in an upregulation of pSmad1/5/8 expression.

explants cultured alone expressed low levels of pSmad1/5/8 in the  $Msx1/2^-$  region and higher levels of pSmad1/5/8 in the  $Msx1/2^+$  domain. In the presence of SU5402, stage 14 OP explants generated cells with upregulated pSmad1/5/8 levels, indicating that, after 24 hours, Bmp activity is increased in the absence of Fgf signals (Fig. 7A,B). This supports our hypothesis that Fgf signaling restricts Bmp activity during the differential specification of sensory and respiratory epithelial cells.

To extend this analysis in vivo, we examined the expression of pSmad1/5/8 in stage 17–21 *Fgfr3c-ΔC*-electroporated chick embryos. During these conditions, pSmad1/5/8 expression was upregulated in olfactory placodal and sensory epithelial cells compared with the non-electroporated control side (Fig. 7C–F), and cells of respiratory character were induced (Fig. 6C,D; data not shown). In addition, in noggin-electroporated stage 17–21 chick embryos, pSmad1/5/8 expression was downregulated compared with the non-electroporated control side (data not shown). Thus, our results suggest that Fgf activity suppresses and restricts the ability of Bmp signals to induce respiratory cell fate in the nasal epithelium. Taken together, our findings provide further evidence that opposing activities of Fgf and Bmp regulate the olfactory sensory versus respiratory epithelial cell fate decision.

## DISCUSSION

Signaling molecules, including Bmps and Fgfs are known to regulate developmental processes by orchestrating genetic programs that determine cell fate and diversity. In this study, we provide evidence that the opposing activities of Fgf and Bmp signals regulate the specification of olfactory sensory and olfactory respiratory epithelial cells. Our results provide evidence that inhibition of the Bmp and Fgf signaling pathways independently disrupts the invagination of the olfactory placode. In addition, Fgf signals promote the generation of sensory epithelial cells and restrict Bmp activity, and Bmp signals promote the specification of respiratory epithelial cells in the nasal epithelium.

Through quail–chick grafting experiments, we show that *Msx1/2* and *Id3* can be used to detect respiratory epithelial cells. Already at the earliest olfactory placodal stage, cells within the olfactory placode are specified as sensory and respiratory epithelial cells, which is consistent with previous statements that the olfactory placode gives rise to both sensory and respiratory epithelial cells (Croucher and Tickle, 1989). Moreover, at early olfactory pit stages in both chick and mouse, separate sensory and respiratory epithelial domains have been formed. Thus, the identification of markers of early respiratory epithelial cells facilitates future studies regarding the development of the nasal epithelium.

Our results suggest a model in which Fgf signals are required for the generation of sensory neural epithelial cells in the nasal epithelium, in part by restricting the ability of Bmp signaling to induce respiratory epithelial cells. In the absence of Fgf activity, both chick and mouse olfactory placodal cells acquire a respiratory non-neural epithelial character. Moreover, the loss of sensory epithelial cells in the nasal epithelium is not caused by a secondary effect of disturbed placodal invagination, but a specific requirement of ongoing Fgf activity. Consistently, previous studies in chick, zebrafish and *Xenopus* have reported that blockage of Fgf signals in prospective neural tissues generates cells of non-neural character (Delaune et al., 2005; Londin et al., 2005; Sjödal et al., 2007; Wilson et al., 2000), supporting the idea that Fgf activity plays an important role in promoting neural cell identity. In agreement with this, Fgf-induced Erk1/2 signaling is required for the specification of neural cells (Stavridis et al., 2007).

Relatively few signaling pathways act during embryonic development and their time-point of action is crucial for the outcome of cell fate choices. At gastrula stages, Bmp signals specify both olfactory and lens placodal cells in anterior neural plate border cells (Sjödal et al., 2007). Somewhat later, at neural fold stages, sustained Bmp activity in prospective olfactory and lens placodal cells inhibits the generation of olfactory placodal cells and promotes lens fate (Sjödal et al., 2007). We now provide evidence that in both chick and mouse at olfactory placodal and pit stages, Bmp activity is required for the generation of respiratory non-neural epithelial cells and, in the absence of Bmp activity, olfactory placodal cells acquire a sensory epithelial progenitor fate. In line with this, enhanced Bmp activity in olfactory placodal and prospective sensory epithelial cells prior to stage 21 induces respiratory non-neural epithelial fate. Thus, Bmp signals specify different cell fates in the olfactory region in a cell-stage-dependent manner.

In agreement with our finding that Bmp activity inhibits the generation of sensory epithelial cells, addition of Bmp4 blocked the formation of neuronal colonies in a mouse olfactory sensory cell line assay (Shou et al., 1999). Although Shou and colleagues concluded that Bmp activity inhibits neurogenesis (Shou et al., 1999), in another study using the same olfactory sensory cell line assay, they suggested that Bmp signals are required for neurogenesis (Shou et al., 2000). However, the acquired cell fates were not determined in these studies. Our findings reconcile these seemingly contradictory results by providing evidence that, at early olfactory placodal stages, Bmp activity inhibits neurogenesis by inducing respiratory epithelial cells. Moreover, in the absence of Bmp activity, the differentiation of olfactory sensory neurons is suppressed but not completely eliminated, and most olfactory placodal cells acquire sensory

epithelial progenitor character. Although Bmp signals are not essential for the generation of olfactory neurons, Bmp activity plays a role in promoting neurogenesis in the nasal epithelium. Similar results are observed in the hippocampus of the mouse, where inhibition of Bmp activity increases precursor cell populations (Bonaguidi et al., 2008). In addition, a recent study demonstrated that Bmp signals are required for neurogenesis in the adult mouse subventricular zone (Colak et al., 2008). Collectively, our work and previous studies suggest a conserved molecular mechanism for the control of neurogenesis in the olfactory sensory epithelium, the hippocampus and the subventricular zone. In these regions, neurogenesis persists in adulthood, substantiating the use of the olfactory sensory epithelium as a model system in examining processes of neurogenesis.

In both chick and mouse, the lack of Fgf or Bmp activity in olfactory placodal cells severely disrupts invagination of the placode, in part by reducing cell proliferation. These results indicate that the initiation of placodal invagination might be related to cell number. Collectively, our results suggest a conserved mechanism in amniotes, in which Fgf and Bmp signals are required for the invagination of the olfactory placode. Consistently, mice deficient in *Fgf8* in the olfactory placodes lack, or have severely reduced, olfactory placodes and nasal cavities (Kawauchi et al., 2005). A similar finding has been described in a recent study, in which inhibition of Fgf activity in the chick otic placodal region resulted in lack of otic placodal invagination (Sai and Ladher, 2008). However, additional studies describing the molecular mechanisms regulating placodal invagination are required to understand this process in more detail. As the fate of cells in the remaining olfactory epithelium differs depending on whether Bmp or Fgf activity is inhibited, our results provide evidence that the specification of sensory and respiratory epithelial cells is independent of morphological movements related to invagination.

Nasal epithelial cells can switch between a sensory and respiratory epithelial cell fate in response to Fgf and Bmp activity. The idea that Fgf and Bmp signals act in an opposing manner during cell specification has previously been revealed in various tissues (De Robertis and Kuroda, 2004; Minina et al., 2002; Neubuser et al., 1997; Rice et al., 2005; Sjödal et al., 2007; Wilson and Edlund, 2001; Yoon et al., 2006). Our results indicate that Fgf signals restrict the range of Bmp activity in the nasal epithelium, thereby allowing differentiated specification of sensory and respiratory cell fate. Both Fgf inhibition and Bmp activation upregulated pSmad1/5/8 expression, which resulted in induction of respiratory epithelial cells and inhibition of sensory epithelial cells. Accordingly, inhibition of Bmp activity resulted in a decrease of pSmad1/5/8, which promoted the generation of sensory epithelial cells at the expense of respiratory epithelial cells. In line with our findings, recent results have suggested that Fgf-induced *Foxg1* (Storm et al., 2006) promotes the generation of olfactory sensory neurons via upregulation of the Tgfb antagonist follistatin, which antagonizes *Gdf11* activity in the olfactory pit (Kawauchi et al., 2009). In summary, we find that at olfactory placodal and pit stages, Fgf and Bmp signals act in an opposing manner to regulate sensory versus respiratory epithelial cell fate decision. Our results reveal that Fgf signals are required for the generation of sensory epithelial cells and restrict the range of Bmp signals, and that Bmp activity promotes respiratory epithelial character.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

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