

# ***Bmp4*-directed nuclear cyan fluorescent protein provides a tool for live imaging and reveals cellular resolution of *Bmp4* expression patterns during embryogenesis**

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**ABSTRACT** The genesis of hair follicles in mammals involves multiple inductive and suppressive signaling interactions between epithelial cells and the underlying mesenchymal tissue. *Bmp4* (Bone Morphogenetic Protein 4) is expressed in the mesenchymal tissue surrounding developing hair follicles. The BMP signaling pathway is suggested to play a suppressive role in hair follicle induction. In addition, it is also found to be important for specification of different cell types in mature hair follicles. Knowledge of the precise expression pattern of *Bmp4* during hair follicle differentiation should provide insights into how these suppressive and differentiative roles regulate hair follicle development. However, *in situ* hybridization studies do not provide sufficient cellular resolution, and three-dimensional reconstructions of serial sections are tedious. We have targeted a nuclear-localized cyan fluorescent protein (CFP) reporter into the endogenous *Bmp4* locus. Nuclear CFP expression was detected in embryonic tissues in a *Bmp4*-specific pattern, including lateral mesoderm, limb bud, dorsal optic cup, lung bud epithelium, heart outflow tract, otocyst, branchial arches, nasal pits, mammary buds, vibrassa and hair follicles. In developing hair follicles, the nuclear CFP reporter provided precise cellular resolution of *Bmp4* expression patterns. These mice provide a novel tool for visualizing *Bmp4* expression patterns in live and fixed tissues with cellular resolution. In addition, these studies reveal a novel view of the arrangement and dynamic changes of *Bmp4*-expressing cells of different stage hair follicles.

**KEY WORDS:** *BMP4*, fluorescent protein, hair follicle, live imaging

## **Introduction**

Bone morphogenetic proteins (BMPs) are a group of secreted signaling molecules belonging to the transforming growth factor-beta superfamily (Hogan, 1996). BMPs signal through binding of receptor kinase proteins on the cell membrane and trigger transcriptional regulation of downstream effector genes (Derynck and Zhang, 2003). BMP4 is one of the most broadly expressed BMPs and has been implicated to play important roles in an array of biological processes and the genesis of many organ systems (Bitgood and McMahon, 1995, Zhao, 2003). The generation of novel fluorescent protein variants that localize to various subcellular locations provides new opportunities to examine develop-

mental expression patterns (Hadjantonakis and Papaioannou, 2004). Here we report the generation of a new *Bmp4* knock-in allele in the mouse that labels the nuclei of *Bmp4*-expressing cells with a CFP. The CFP expression pattern in *Bmp4*<sup>CFP</sup> mice was found to mimic the known *Bmp4* expression pattern in mouse embryos. The cellular resolution provided by this allele offers a novel view of *Bmp4* expression patterns. Specifically, we used these mice to analyze the cellular pattern of *Bmp4* expression in developing hair follicles of the fetal mouse skin. The dynamic and

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*Abbreviations used in this paper:* BMP, bone morphogenetic protein; CFP, cyan fluorescent protein.

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reiterative changes in the pattern of *Bmp4*-expressing cells in developing hair follicles suggest complex roles for BMP4 at different stages of hair follicle development.

## Results

### Generation of a nuclear cyan fluorescent protein (CFP) knock-in allele of *Bmp4* in the mouse

We originally designed the *Bmp4* knock-in allele to simultaneously mark the cell nuclei of *Bmp4*-expressing cells and the secreted BMP4 ligand with different fluorescent proteins and observe the structure of BMP4 morphogen gradients *in vivo*. Therefore, we targeted the endogenous *Bmp4* allele with a gene construct comprising two different fluorescent proteins in mouse embryonic stem (ES) cells (Fig. 1A). In the targeted allele, a human histone H2b-fused CFP gene is inserted at the endogenous translational start codon. The expressed histone H2b-fused fluorescent protein (FP) will be incorporated into chromatin and serves as a nuclear and mitotic chromosome marker (Hadjantonakis and Papaioannou, 2004). A yellow fluorescent protein (YFP) is inserted in frame into the coding sequence of BMP4 after the furin cleavage site, so that the

TABLE 1

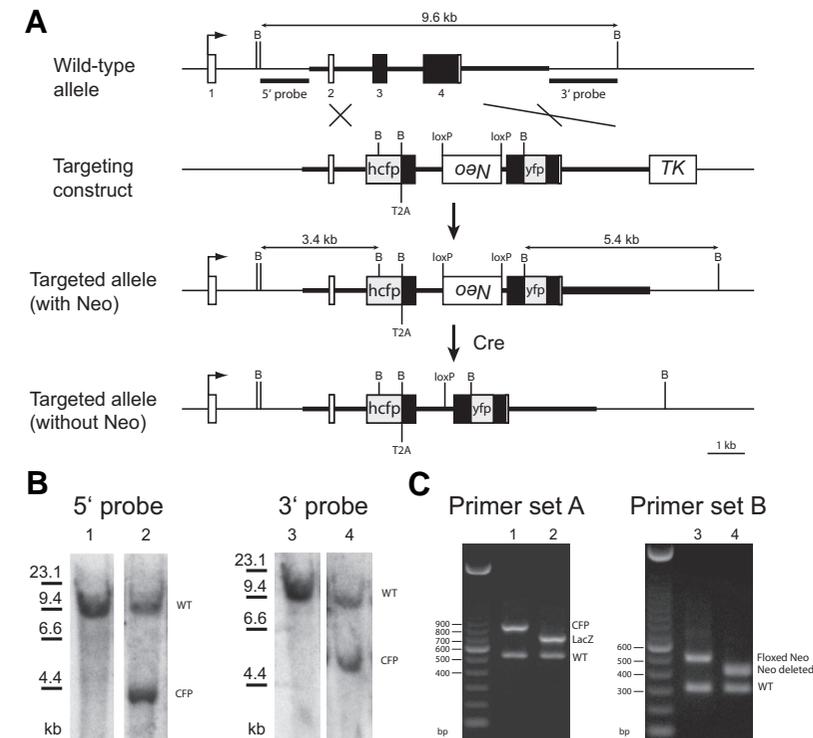
### GENETIC TEST OF *BMP4*<sup>CFP</sup> ALLELE FUNCTION

Parental Genotypes	Number of Live Born Pups of Specific Genotypes			
	<i>Bmp4</i> <sup>CFP/+</sup>	<i>Bmp4</i> <sup>LacZ/+</sup>	<i>Bmp4</i> <sup>CFP/+</sup>	<i>Bmp4</i> <sup>LacZ/CFP</sup>
<i>Bmp4</i> <sup>CFP/+</sup> × <i>Bmp4</i> <sup>LacZ/+</sup> (a)	14	14	13	0
<i>Bmp4</i> <sup>CFP/+</sup> × <i>Bmp4</i> <sup>CFP/+</sup> (b)	15	26	0	0

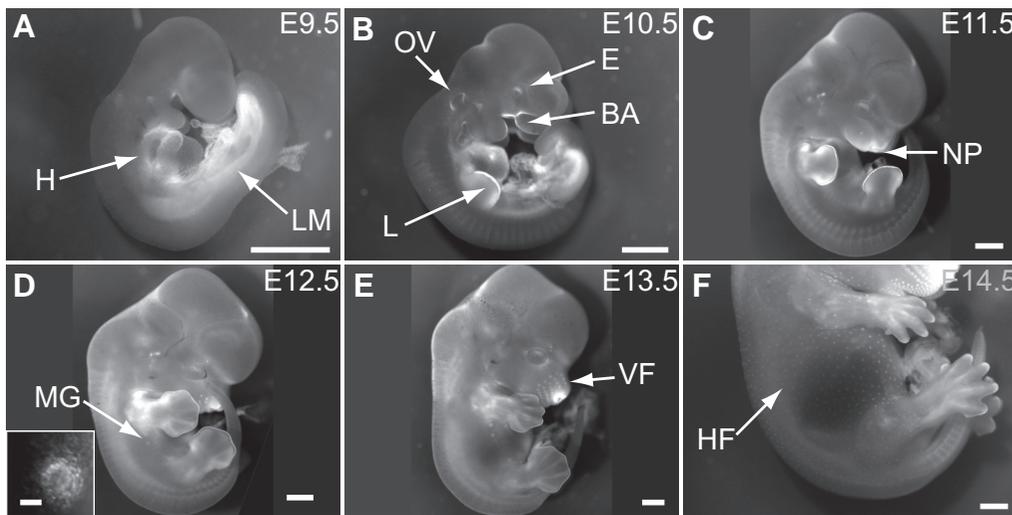
(a) Total n = 40 from 7 litters. (b) Total n = 41 from 6 litters.

mature BMP4 peptide will have an N-terminal YFP tag. A similar fluorescent protein tagging strategy was used for the *Drosophila* BMP4 orthologue Dpp to observe morphogen gradients in the wing maginal disc by overexpression of the chimeric protein (Teleman and Cohen, 2000). Between the H2b-CFP and BMP4-YFP coding sequences, a self-cleavage viral 2A peptide sequence was incorporated to mediate co-expression and post-translational separation of the two gene products (Szymczak and Vignali, 2005). Thus, *Bmp4*-expressing cells should be marked in their nuclei with CFP and the BMP4 ligand tagged with YFP should be secreted and function as a wild-type allele.

We successfully targeted the construct into the endogenous locus in ES cells and generated mice bearing the allele named *Bmp4*<sup>CFP</sup> (Fig. 1B and C). The neomycin resistance (neo) expression cassette was then removed by crossing to the *Sox2-Cre* mouse line (Hayashi et al., 2003), and all experiments were conducted on mice with the neo cassette removed. Unfortunately, *Bmp4*<sup>CFP</sup> proved to be a functionally null allele. *Bmp4*<sup>LacZ</sup> is a null allele that has part of the *Bmp4* coding region replaced by a beta-galactosidase reporter gene (Lawson et al., 1999). We were unable to recover any live born animals with a *Bmp4*<sup>CFP/LacZ</sup> genotype in crosses between *Bmp4*<sup>CFP/+</sup> and *Bmp4*<sup>LacZ/+</sup> animals (Table 1). In addition, we could not recover any live born *Bmp4*<sup>CFP/CFP</sup> animals in intercrosses of *Bmp4*<sup>CFP/+</sup> mice (Table 1). *Bmp4*<sup>CFP/CFP</sup> embryos show growth retardation at E9.5 (embryonic day 9.5) (Fig. 1E), resembling the homozygous *Bmp4* null phenotype (Winnier et al., 1995). Although CFP expression was readily observed in *Bmp4*<sup>CFP/+</sup> mice (see below), no YFP fluorescence was detected, suggesting that undetectable levels of BMP4 are produced from the targeted allele. However, *Bmp4*<sup>CFP/+</sup>



**Fig. 1. Generation of *Bmp4*<sup>CFP</sup> knock-in allele and homozygous phenotype.** (A) Gene targeting strategy. The wild-type *Bmp4* allele contains four exons, labeled by numbers below. The floxed Neo cassette is removed by *Cre* expression in the mouse germ line. A 5' probe and a 3' probe are used to identify correctly targeted ES cell clones. *Hcfp*: histone H2b-CFP; *Neo*: P<sub>gk</sub>-Neo-bpA positive selection cassette; *TK*: MC1-thymidine kinase negative selection cassette; *B*: BamHI sites. (B) Southern analysis of BamHI-digested genomic DNA isolated from ES cells. Lanes 1 and 3, wild-type controls, lanes 2 and 4 correctly targeted clone (clone 2C9) used to generate germline chimeras. (C) PCR genotyping results of tail DNA. Primer set A allows for differentiation between wild-type, CFP and LacZ alleles. Lanes 1 and 2, *Bmp4*<sup>CFP/+</sup> and *Bmp4*<sup>LacZ/+</sup> animals, respectively. Primer set B allows for detection of the deletion of Neo cassette in the targeted allele. Lanes 3 and 4, heterozygous animals carrying one CFP allele before and after *Cre*-mediated deletion of the Neo selection cassette, respectively. (D) E9.5 wild-type embryo. (E) E9.5 *Bmp4*<sup>CFP/CFP</sup> embryo, showing growth retardation. Scale bar, 1 mm.



**Fig. 2. CFP signal observed in known *Bmp4*-expressing tissues.** Different stage *Bmp4*<sup>CFP/+</sup> heterozygous embryos imaged under fluorescent stereo microscope with CFP filters. The embryos are at E9.5 (A), E10.5 (B), E11.5 (C), E12.5 (D), E13.5 (E), E14.5 (F) stages. Arrows point to the CFP signal positive tissues/organs that are known to express *Bmp4*. BA, branchial arches; E, eye; H, heart; HF, hair follicle; L, limb; LM, lateral mesoderm; MG, mammary gland; NP, nasal pit; OV, otic vesicle; VF, vibrissa follicle. Scale bars, 1 mm. Insert in (D) shows a higher magnification view of mammary gland; scale bar, 100  $\mu$ m.

heterozygous animals appear normal and are fertile. Hence, we focused our study on the nuclear CFP signal patterns in the heterozygous animals.

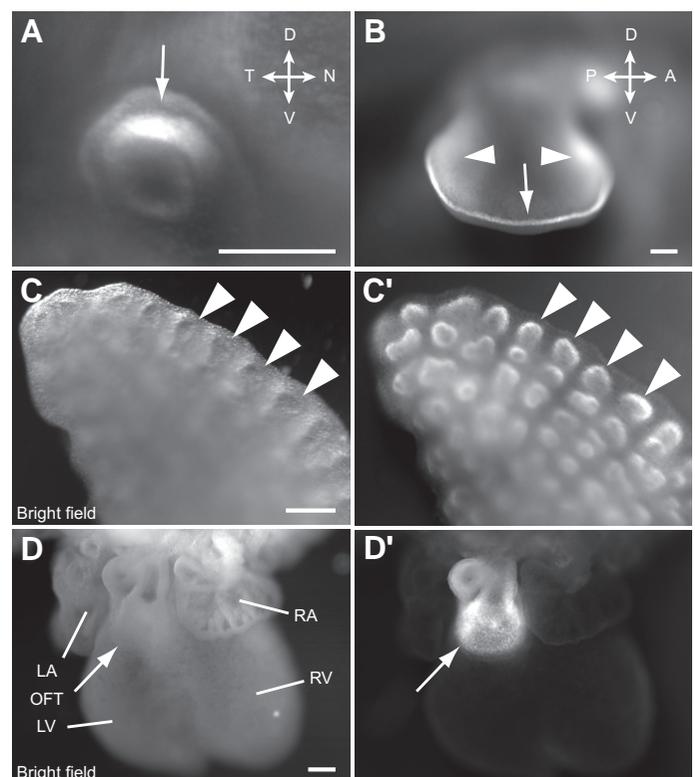
#### CFP expression patterns in *Bmp4*<sup>CFP/+</sup> are consistent with *Bmp4* expression

Because H2b-CFP has been inserted into the *Bmp4* locus, expression should be under the control of the endogenous *Bmp4* regulatory elements. Thus, the fluorescent signal in *Bmp4*<sup>CFP/+</sup> animals should reflect the endogenous *Bmp4* expression pattern and agree with expression studies observed by other methods, i.e. *in situ* hybridization or X-gal staining with a *lacZ* knock-in allele (Lawson *et al.*, 1999). We examined whole embryos or specific fetal organs at different stages and observed CFP signals in the known *Bmp4*-specific expression patterns.

Prior to E9.5, CFP signals in *Bmp4*<sup>CFP/+</sup> embryos were either undetectable or very weak. In the E9.5 *Bmp4*<sup>CFP/+</sup> embryo, the CFP signal is strong in the lateral mesoderm and the developing heart (Fig. 2A), in agreement with a previous report (Jones *et al.*, 1991). In the early stage limb bud, *Bmp4* is expressed in both the mesoderm and the apical ectodermal ridge (AER), and, together with other BMPs, plays key roles in skeletal genesis and patterning in the limb (Bandyopadhyay *et al.*, 2006, Maatouk *et al.*, 2009, Selever *et al.*, 2004). The CFP signal in the E10.5–11.5 *Bmp4*<sup>CFP/+</sup> embryo shows strong expression in the AER and the broader expression domains in the limb mesoderm (Fig. 2B and 3B). *Bmp4* was also shown to be essential for patterning and development of the eye (Behesti *et al.*, 2006, Furuta and Hogan, 1998). In the E10.5 *Bmp4*<sup>CFP/+</sup>

embryo, the CFP signal is present in the eye and is restricted to the dorsal domain of the optic cup, reconfirming the known *Bmp4* expression pattern (Fig. 2B and 3A). In the developing lung, *Bmp4* is expressed in the epithelium at the tip of lung buds and plays a key role in branching morphogenesis (Bellusci *et al.*, 1996, Que *et al.*, 2006). The CFP signal in the E13.5 *Bmp4*<sup>CFP/+</sup> embryo recapitulates the specific *Bmp4* expression pattern in the embryonic lung (Fig. 3C'). In the developing heart, *Bmp4* is expressed in the outflow tract myocardium and endocardial cushion of outflow tract ridges in E12–14 stage embryos and plays an essential role in heart formation (Abdelwahid *et al.*, 2001, McCulley *et al.*, 2008). The CFP signal in the E13.5 *Bmp4*<sup>CFP/+</sup> embryo highlights the outflow tract in the E13.5 heart (Fig. 3D'). In addition, the CFP pattern

**Fig. 3. CFP signal shows specific *Bmp4* expression patterns in developing organs.** (A) E10.5 eye. CFP is detected in the dorsal optic cup (arrow). (B) E11.5 forelimb bud, CFP is expressed in the AER (arrow) and the mesodermal regions (arrowheads). (C) Brightfield image of E13.5 lung. (C') CFP view of (C). CFP is specifically elevated in the epithelium cells at the tip of lung buds (arrow heads). (D) Brightfield image of E13.5 heart. (D') CFP view of (D). CFP is expressed in the outflow tract arrow. LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. Orientation: D, dorsal; Vm, ventral; N, nasal; T, temporal; A, anterior; P, posterior. Scale bars, 200  $\mu$ m.

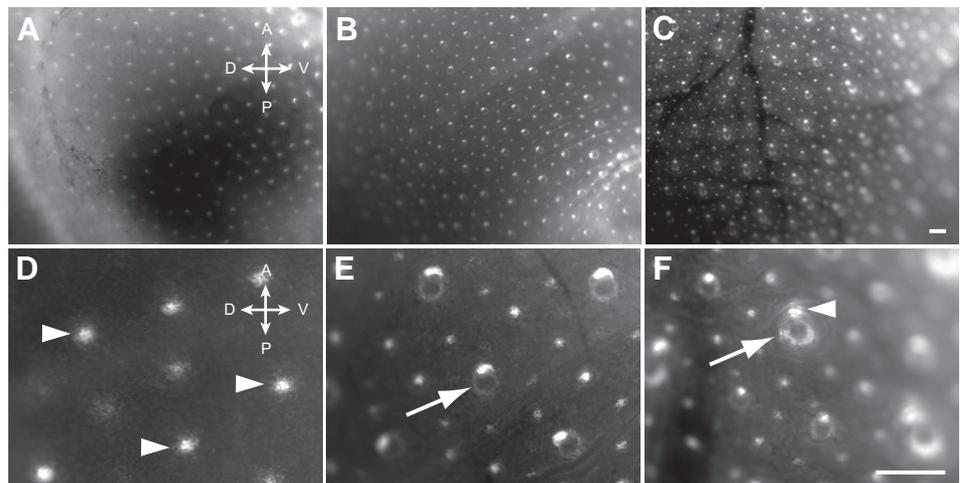


in *Bmp4<sup>CFP/+</sup>* embryos also reflects several *Bmp4* expression domains that have been reported, including the otocyst (Fig. 2B) (Grotewold *et al.*, 2001), branchial arches (Fig. 2B) (Grotewold *et al.*, 2001), nasal pit (Fig. 2C) (Jones *et al.*, 1991), mammary buds (Fig. 2D) (Phippard *et al.*, 1996), vibrissa follicles (Fig. 2E) (Jones *et al.*, 1991, Ozeki *et al.*, 2004), and hair follicles (Fig. 2F) (Botchkarev *et al.*, 1999).

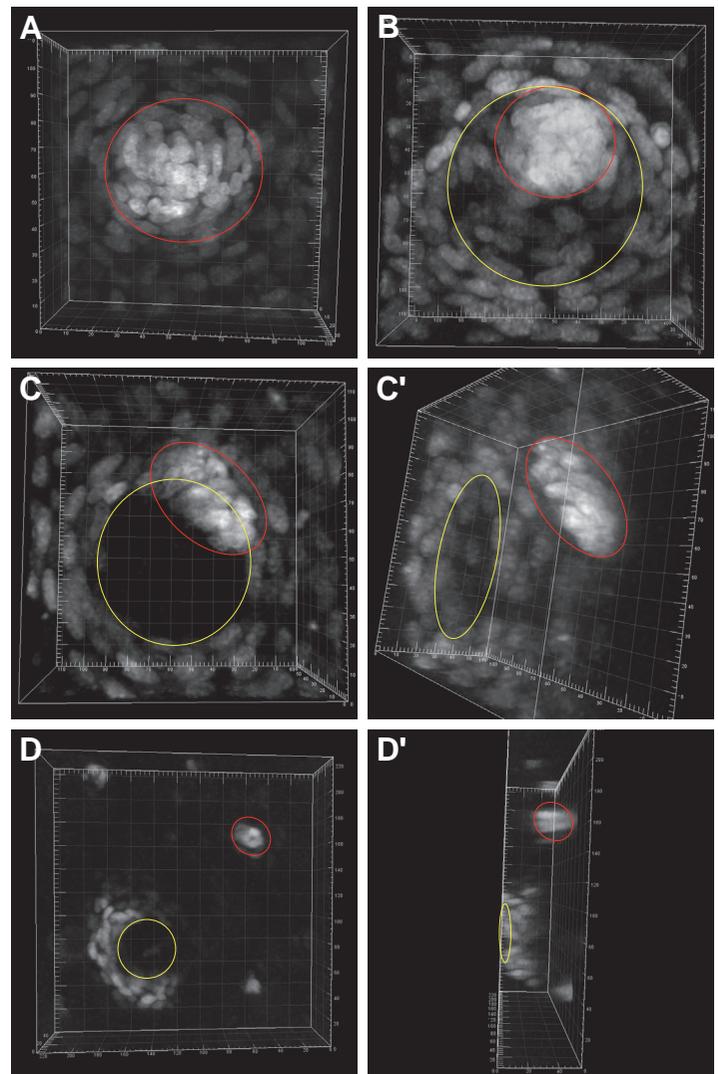
#### Cellular resolution *Bmp4* expression patterns in developing hair follicles

The development of hair follicles involves complex signaling mechanisms between adjacent tissues, including Wnts (Andl *et al.*, 2002, Reddy *et al.*, 2001), Shh (St-Jacques *et al.*, 1998), Edar (Headon and Overbeek, 1999, Mikkola *et al.*, 1999) and BMPs (Botchkarev and Sharov, 2004), that have been implicated in the initiation, patterning or differentiation of hair follicles. These signaling molecules and some of their antagonists need to be expressed in proper domains and at correct time points for normal hair follicle formation. (Schmidt-Ullrich and Paus, 2005).

Taking advantage of the nuclear localized CFP signal in the *Bmp4<sup>CFP/+</sup>* mice that provides precise information of *Bmp4*-expressing cells, we performed a detailed analysis of the *Bmp4* expression pattern at different stages of hair follicle development in the embryo. The CFP signal in the ectoderm is initiated between E13.5 and E14.5, forming focal spots in E14.5 embryos (Fig. 2F, 4A and 4D). This timing correlates with the onset of primary hair placode formation (Schmidt-Ullrich and Paus, 2005). On the skin of E15.5 embryos, the CFP signal outlines many oval-shaped structures with an accompanying brighter spot asymmetrically on one side of each oval ring, and the rings are evenly spaced and surrounded by small focal spots (Fig. 4B and 4E). These ovals with their associated asymmetric spots are coordinately oriented in the same direction. In E16.5 embryos, the CFP signal continues to form more rings with spots, while some bigger rings have their accompanying spot apparently moving further away from the ring (Fig. 4C and 4F). In later stage embryos, the CFP signal basically repeats similar patterns as that in E16.5 but with more foldings of the skin as the epithelium thickens (data not shown). With confocal microscopy, we observed the nuclear CFP pattern at sub-



**Fig. 4. *Bmp4* expression pattern in the skin changes through different developmental stages.** E14.5 (A,D), E15.5 (B,E) and E16.5 (C,F) skin. At E14.5, CFP signal is first observed on the skin as evenly distributed focal spots (arrowheads in D). At E15.5, the ring and spot structures form (arrow in E). At E16.5, the spot (arrowhead in F) associated with a ring is further away from the ring in more mature follicles (arrow in F). Scale bars, 200  $\mu$ m. (A-C) are on the same scale; (D-F) are on the same scale.



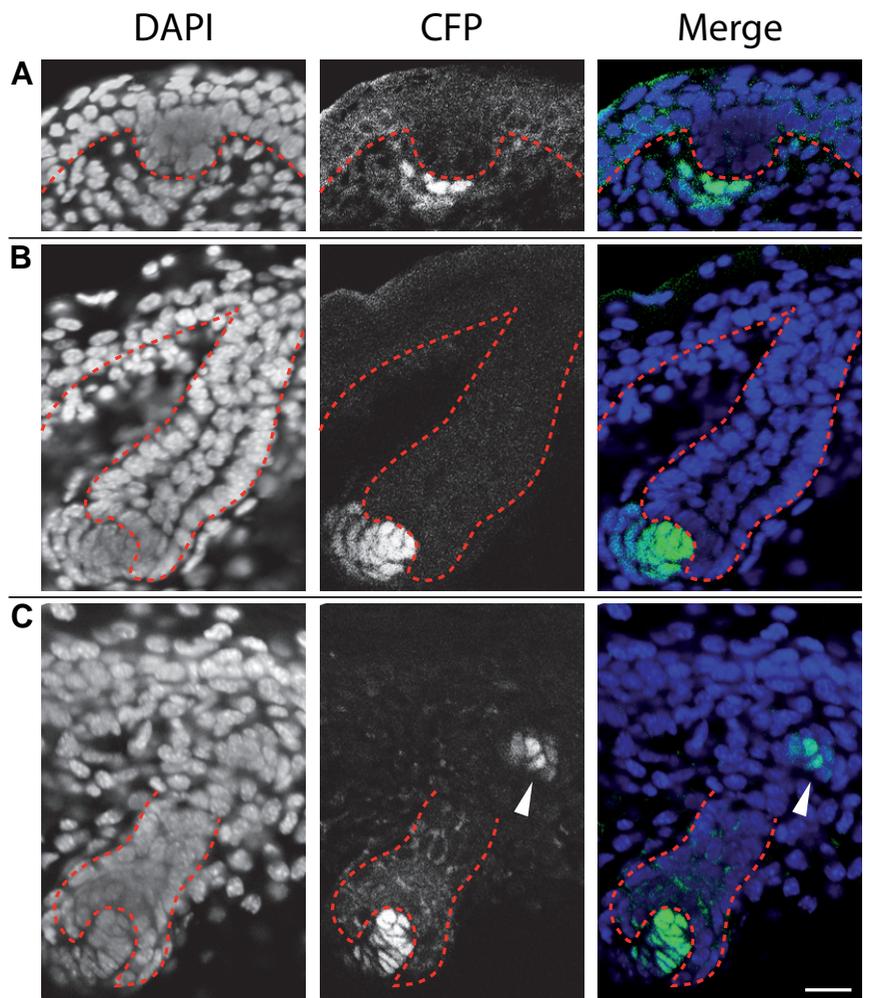
**Fig. 5. *Bmp4* expressing cells form different structures on the skin at different developmental stages.** Three-dimensionally reconstructed confocal images of CFP signals provide cellular resolution on *Bmp4* expression pattern at different stages. (A) At E14.5, *Bmp4*-expressing cells form focal spots (red circle) with cells in the center fluorescing more strongly. (B,C) At E15.5, the *Bmp4*-expressing cells form the ring (yellow circle) and spot (red circle) structures. (D) In more mature hair follicle of E16.5 skin, the bright spot (red circle) appears to move further away from the ring (yellow circle). (A,B,C,D) Views onto the surface of the skin, whereas (C') is a tilted view of (C), and (D') is a 90 degree rotated view of (D). The spot is actually located beneath the ring in the ring and spot structures, as shown in (C',D'). Ruler numbers indicate length in  $\mu$ m.

cellular resolution of in live fetal skin and reconstructed three dimensional images. In E14.5 skin, the expression is concentrated in an aggregate of cells, with cells having stronger fluorescence located at the center of every focal spot (Fig. 5A). In E15.5 skin, a ring structure forms with a central region of non-expressing cells surrounded by expressing cells and a group of strongly fluorescing cells residing on one side of the ring (Fig. 5B and 5C). The group of cells with strong signal is actually located beneath the ring of structure, revealed by rotating the reconstructed three-dimensional images ~45 degrees (Fig. 5C'). In E16.5 skin, the bright spot is farther away from the ring (Fig. 5D and 5D'). We also generated videos rotating the reconstructed images for a better understanding of the structures formed by *Bmp4*-expressing cells (Supplementary Materials Online). To know the positional relationship of these *Bmp4*-expressing cells to the developing hair follicles, we made frozen sections of fixed skin tissue. In early stage hair placodes, the *Bmp4*-expressing cells are in the mesenchymal layer subjacent to the hair placode (Fig. 6A). In more mature hair follicles, *Bmp4* is expressed in the dermal papilla of involuted hair follicles (Fig. 6B). The *Bmp4*-expressing dermal papilla cells eventually become surrounded by non-expressing cells in the bulb structure in late stage hair follicles (Fig. 6C).

## Discussion

We have generated a novel *Bmp4* allele that expresses a nuclear-localized CFP reporter for live imaging and cellular resolution of *Bmp4* expression patterns. The observations of CFP signals in various tissues compared with the known pattern of *Bmp4* expression show that the CFP signal faithfully reflects the expression pattern of *Bmp4*. Thus, the *Bmp4*<sup>CFP/+</sup> mice can be used as an imaging tool to visualize *Bmp4* expression patterns in live tissues. In addition, *Bmp4*<sup>CFP/+</sup> mice provide a novel resource for the isolation of *Bmp4*-expressing cells in live tissues using gross dissection or flow cytometry for the analysis of gene expression profiles or biochemical studies. However, the fluorescent signal is very weak at E8.5 and almost undetectable in E7.5 and earlier stage embryos, although *Bmp4* expression is detected at these time points by *in situ* hybridization or beta-galactosidase detection using the *lacZ* knock-in allele (Lawson *et al.*, 1999, Winnier *et al.*, 1995). However, both *in situ* and beta-galactosidase staining techniques detect enzymatically amplified signals and are very sensitive, whereas with the *Bmp4*<sup>CFP</sup> allele, the signal strength largely depends on absolute level of fluorescent protein produced and is especially prone to photobleaching at low levels.

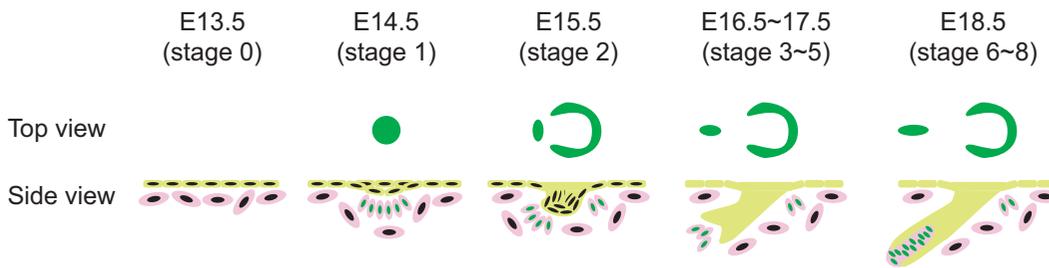
We were surprised that the *Bmp4*<sup>CFP</sup> allele is functionally a null or very severe hypomorphic allele because *in vitro* studies of the BMP4-YFP fusion protein in transient tissue culture assays or *Xenopus* embryos suggested that the fusion protein was active



**Fig. 6. *Bmp4* expressing cells associate with developing hair follicles.** Frozen sections of E15.5 (A), E16.5 (B) and E18.5 (C) skin show the respective localization of *Bmp4*-expressing cells by CFP signals in developing hair follicles. The epithelial layer and hair follicles are outlined by red dashed lines. (A) *Bmp4* is first expressed by mesenchymal cells (arrow) underneath the hair placode. (B,C) At later stages, the dermal papillae express *Bmp4* (arrows). Weaker CFP positive cells are seen near the base of hair follicles (arrowhead in C).

(Jang, Ueno, and Behringer, unpublished observations). In our targeting strategy, no endogenous *Bmp4* sequence was deleted in the targeted allele, so that all of the *cis*regulatory sequences are present. Integration of exogenous sequences into an endogenous locus with exons and introns is different from cDNA expression from plasmids and expression of synthetic mRNA. We speculate that our generation of a null allele may have to do with expression and/or mRNA processing from a genomic locus. It is possible that the YFP-tagged BMP4 protein is destabilized *in vivo*, and this stability issue was masked by overexpression in cell culture and *Xenopus* embryos. In addition, western blot analysis of protein extracts from tissues of *Bmp4*<sup>CFP/+</sup> embryos shows a very weak or undetectable YFP-BMP4 fusion protein band (data not shown).

We summarize our findings of *Bmp4* expression in the embryonic skin using the *Bmp4*<sup>CFP</sup> allele in Fig. 7. In E13.5 skin, prior to hair placode formation, *Bmp4* expression is not detected. In E14.5 skin, placodes begin to form and *Bmp4* is expressed by mesenchymal cells underneath the placodes. In E15.5 skin, as the



**Fig. 7. Summary of *Bmp4*-expressing cells in different stage hair follicles.** *BMP4* expressing cells are marked by green nuclei. At E14.5, the aggregated *Bmp4*-expressing cells underneath initiating hair placode are seen as spots on the skin. At E15.5, the ring and spot pattern comes from the strong expressing cells be-

low the tip of invaginating hair follicle. At E16.5 and later stages, the *Bmp4*-expressing cells form the dermal papilla and surround the base of hair follicles. The schematic hair follicle developmental stage diagram in this figure is modified from the review by Schmidt-Ullrich and Paus, 2005.

epithelial cells in primary hair follicles start to invaginate, cells with stronger *Bmp4* expression form a cap structure at the tip of the invaginated epithelium structure with a ring of *Bmp4*-expressing cells surrounding the invagination at the base. At E16.5, *Bmp4* is expressed in the dermal papilla adjacent to the tip of hair follicles that are extending further into dermis. At E18.5, *Bmp4* expression is maintained in the dermal papilla cells surrounded in the bulb structure in late stage hair follicles.

The suppressive activity of BMPs on hair placode formation has been implicated in different experimental systems (Botchkarev *et al.*, 1999, Jung *et al.*, 1998, Noramly and Morgan, 1998). In addition, the importance of the BMP signaling pathway in specification of different cell types in the hair follicle has been well documented (Botchkarev and Sharov, 2004). The ring-shaped expression pattern of developing hair follicles is quite unique, among other signaling molecules that are expressed in the hair follicle and required for proper development (Laurikkala *et al.*, 2002, Mou *et al.*, 2006). This may reflect a unique role BMP4 plays or the advantage of using fluorescent protein labeling technique since many gene expression patterns have only been examined on sections on which the three dimensional structure is not easily noticed. It will be interesting to determine how the dynamic *Bmp4*-expressing cell rings and focal aggregates that we have discovered relate to these inductive and differentiation roles of BMP4 during hair follicle development.

Here we have shown the feasibility of visualizing *Bmp4* expression in live tissues with our knock-in allele, and analyzed *Bmp4* expressing domains associated with developing hair follicles. This allele provides a new experimental tool for studying BMP4 signaling mechanisms in live tissues. In addition, BMP reporter transgenic mouse lines that express EGFP in BMP signal-receiving cells have been created (Blank *et al.*, 2008, Monteiro *et al.*, 2008). EGFP and CFP are spectrally distinguishable (Dickinson *et al.*, 2002, Lansford *et al.*, 2001, Zimmermann, 2005). The possibility of combining our *Bmp4*<sup>CFP</sup> allele with the BMP signaling GFP reporter lines may further advance the study of BMP signaling by live tissue imaging techniques.

## Materials and Methods

### Generation of *Bmp4*<sup>CFP</sup> knock-in allele in the mouse

The targeting construct was generated by subcloning a 2.1-kb 5' homologous region upstream from the endogenous start site of *Bmp4* to fuse with a human histone H2b-Cerulean (an improved CFP) coding sequence (Hadjantonakis and Papaioannou, 2004, Rizzo *et al.*, 2004), followed by a self-cleavage 2A sequence from the virus *Thosea asigna* (Szymczak and Vignali, 2005), followed by the endogenous coding sequence for BMP4 as an in-frame fusion. A floxed Pgk-neo-bpA cassette

[from pL452 (Liu *et al.*, 2003)] was inserted into the Eco RI site into the preserved third intron in reverse orientation. An improved YFP, Citrine (Griesbeck *et al.*, 2001), coding sequence was inserted into the fourth exon as an in-frame fusion after the furin cleavage site that generates the mature peptide. An additional homologous region of 3.3 kb was included in the targeting construct after the Citrine coding sequence. In our targeting strategy, no endogenous sequence was deleted. An MC1-TK cassette was placed next to the 3' homologous region for negative selection. G4 ES cells (George *et al.*, 2007) were electroporated with linearized targeting construct DNA and subjected to G418 and FIAU double drug selection. Surviving clones were screened by Southern blotting with *Bam*HI enzyme digestion of genomic DNA. A 1-kb 5' probe and a 1.4-kb 3' probe outside of the homologous region on the targeting construct were used to identify correctly targeted clones. One of these clones was expanded and injected into C57Bl/6J blastocysts to generate chimeras. The floxed Neo cassette was removed through crossing with *Sox2-Cre* transgenic animals (Hayashi *et al.*, 2003). Heterozygous animals carrying the CFP knock-in *Bmp4* allele without Neo are maintained on an outbred SWISS Webster (Taconic, NY) genetic background and used for analysis in this report.

Primers for PCR-genotyping animals:

Set A: BMP4-F1: 5'-GAAGCCACGCTGAGATCATGGC;

BMP4-R1: 5'-CATCTGTAGAAGTGTCCCTCGAAG;

CFP-R: 5'-CGGACACGCTGAACTTGTGG;

LacZ-R: 5'-ACGACAGTATCGGCCTCAGGAAG.

Set B: BMP4-F2: 5'-GCTAGCTGCTCCACAACCTACTCTC;

BMP4-R2: 5'-TGCCGAGGAGATCACCTCATTCTC;

Pgk-R1: 5'-CACCAAGAACGGAGCCGGTTG.

PCR genotyping with Primer Set A with 60.5 °C annealing temperature generates a 550-bp product from the wild-type *Bmp4* allele, a 700-bp product from the null *lacZ* knock-in allele (Lawson *et al.*, 1999), or an 850-bp product from the CFP knock-in allele. PCR genotyping with Primer Set B with 55 °C annealing temperature generates a 320-bp product for the wild-type allele, a 500-bp product for the floxed Neo cassette in the CFP allele, or a 400-bp product for the footprint *loxP* site after Neo is removed by Cre recombinase.

### Imaging of CFP expression patterns in live and fixed tissues

For live imaging of whole embryos, embryos of desired stages were isolated from the uterus of pregnant females from timed matings. Embryos were immersed in phosphate buffered saline (PBS) and imaged at low magnification with a Carl Zeiss Lumar fluorescent stereo microscope system (<http://www.zeiss.de/>). Lungs and heart were dissected from E13.5 embryos for imaging with the same settings. For higher resolution CFP confocal images of the skin, live embryos were placed on a glass bottom dish with PBS and imaged with a Zeiss LSM 510 META laser confocal microscopy system, using a C-Apochromat 40X/W, NA=1.2 Corr objective (Carl Zeiss Inc.). Three-dimensional images were reconstructed using the image processing software Imaris 5.0.3 (Bitplane, St. Paul, MN, USA) (<http://www.bitplane.com/>).

For frozen sections of skin, whole embryos were fixed in 4% paraformaldehyde in phosphate buffered saline for one hour and washed through

10%, 20% and 30% sucrose solutions, and then embedded in O.C.T. compound [Tissue-Tek (<http://www.tedpella.com/>)]. Sections were mounted with DAPI containing medium and then imaged with the same laser confocal microscopy system and objective as that for live skin imaging.

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