Testing Chemotherapeutic Agents in the Feather Follicle Identifies a Selective Blockade of Cell Proliferation and a Key Role for Sonic Hedgehog Signaling in Chemotherapy-Induced Tissue Damage

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Chemotherapeutic agents induce complex tissue responses *in vivo* and damage normal organ functions. Here we use the feather follicle to investigate details of this damage response. We show that cyclophosphamide treatment, which causes chemotherapy-induced alopecia in mice and man, induces distinct defects in feather formation: feather branching is transiently and reversibly disrupted, thus leaving a morphological record of the impact of chemotherapeutic agents, whereas the rachis (feather axis) remains unperturbed. Similar defects are observed in feathers treated with 5-fluorouracil or taxol but not with doxorubicin or arabinofuranosyl cytidine (Ara-C). Selective blockade of cell proliferation was seen in the feather branching area, along with a downregulation of *sonic hedgehog* (*Shh*) transcription, but not in the equally proliferative rachis. Local delivery of the Shh inhibitor, cyclopamine, or *Shh* silencing both recapitulated this effect. In mouse hair follicles, those chemotherapeutic agents that disrupted feather formation also downregulated *Shh* gene expression and induced hair loss, whereas doxorubicin or Ara-C did not. Our results reveal a mechanism through which chemotherapeutic agents damage rapidly proliferating epithelial tissue, namely via the cell population–specific, *Shh*-dependent inhibition of proliferation. This mechanism may be targeted by future strategies to manage chemotherapy-induced tissue damage.

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INTRODUCTION

Chemotherapy often damages normal tissue as an adverse effect. Commonly used chemotherapeutic agents such as cyclophosphamide (CYP), 5-fluorouracil (5-FU), taxol, and doxorubicin (Dox) are believed to be toxic to all mitotic cells. Chemotherapy-induced alopecia (CIA) is common in human patients (Hesketh *et al.*, 2004; Yun and Kim, 2007; Trueb, 2009; Chon *et al.*, 2012), and rodent models have been developed to study the molecular mechanism of chemotherapy-induced tissue damage (Hussein *et al.*, 1990; Botchkarev *et al.*, 2000; Sharov *et al.*, 2004; Jimenez *et al.*, 2008; Paus *et al.*, 1994; 2013). A p53-dependent apoptosis program, which likely involves Fas-dependent signaling events, was suggested to underlie CIA (Botchkarev *et al.*, 2000; Sharov *et al.*, 2004). Other molecules such as ectodysplasin A receptor, epidermal growth factor receptor, and the eukaryotic initiation factor 4E have also been implicated (Brosh *et al.*, 2010; Bichsel *et al.*, 2013; Nasr *et al.*, 2013; Paik *et al.*, 2013).

It is likely that cells interact with chemotherapeutic agents in complex ways. For example, in addition to undergoing apoptosis, cells may ''slip'' out of the cell cycle and thus escape apoptosis (Gascoigne and Taylor, 2008). Also, cell-to-cell variation in response to apoptosis-inducing agents can arise from transcriptional differences (Spencer *et al.*, 2009; Ooi and Ma, 2013). Furthermore, because of the diverse mechanisms of action, it is difficult to rationally predict whether a specific chemotherapeutic agent will cause CIA in a given patient (Hesketh *et al.*, 2004; Yun and Kim, 2007;

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Abbreviations: 5-FU, 5-fluorouracil; Ara-C, arabinofuranosyl cytidine; BrdU, 5-bromo-2-deoxyuridine; CIA, chemotherapy-induced alopecia; CYP, cyclophosphamide; Dox, doxorubicin; γ-H2AX, phosphorylated histone H2AX; LCAM, liver cell adhesion molecule; NCAM, neural cell adhesion molecule; PARP, poly ADP-ribose polymerase; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; qPCR, quantitative PCR; RT-PCR, reverse transcription-PCR; Shh, sonic hedgehog; TEM, transmission electron microscopy

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Trueb, 2009, Chon *et al.*, 2012; Paus *et al.*, 2013). Detailed *in vivo* investigation in appropriate model systems therefore is required to evaluate the alopecia-inducing potential of each specific drug.

In this context, we hypothesized that fundamental and translationally relevant mechanistic insights might be won from studying the feather follicle. Like its distant mammalian relative, the hair follicle, this characteristic avian skin appendage represents a prototypic, cyclically regenerating miniorgan in which complex ectodermal–mesodermal interactions and stem cell biology can be studied exemplarily (Chuong 1998; Lin *et al.*, 2013). Structurally, feathers display a regular branching epithelium (barbs) attached to a central axis (rachis), with smaller branches (barbules) protruding from each barb (Lin *et al.*, 2013; Yu *et al.*, 2004; Prum and Williamson, 2001; Figure 1a–c). The formation of these structures is regulated by a set of evolutionarily conserved molecular pathways. BMP/Noggin and Shh signaling are involved (Yu *et al.*, 2002; Harris *et al.*, 2002; Chang *et al.*, 2004), and a Wnt signaling gradient controls the orientation of the branching (Yue *et al.*, 2006). The same signaling pathways have long been known to be key players in hair follicle development and cycling (St-Jacques *et al.*, 1998; Chiang *et al.*, 1999; Botchkarev *et al.*, 2001; Plikus *et al.*, 2008, 2011; Schneider *et al.*, 2009).

Thus, as long as any relevance of concepts emanating from feather model–based research for hair research can be documented, the feather follicle may become an as yet untapped resource for dissecting selected molecular aspects of CIA pathology. Here, we have tested the impact of chemotherapeutic agents on the developing and cycling feather follicle and have compared this with murine CIA.

RESULTS

CYP treatment induces defects in feather formation

Active feather growth was induced in 6-month-old adult chicken by plucking and regeneration (Chen *et al.*, 2014). Adapting the established protocol of inducing complete alopecia in mice by CYP (Paus *et al.*, 1994), we found

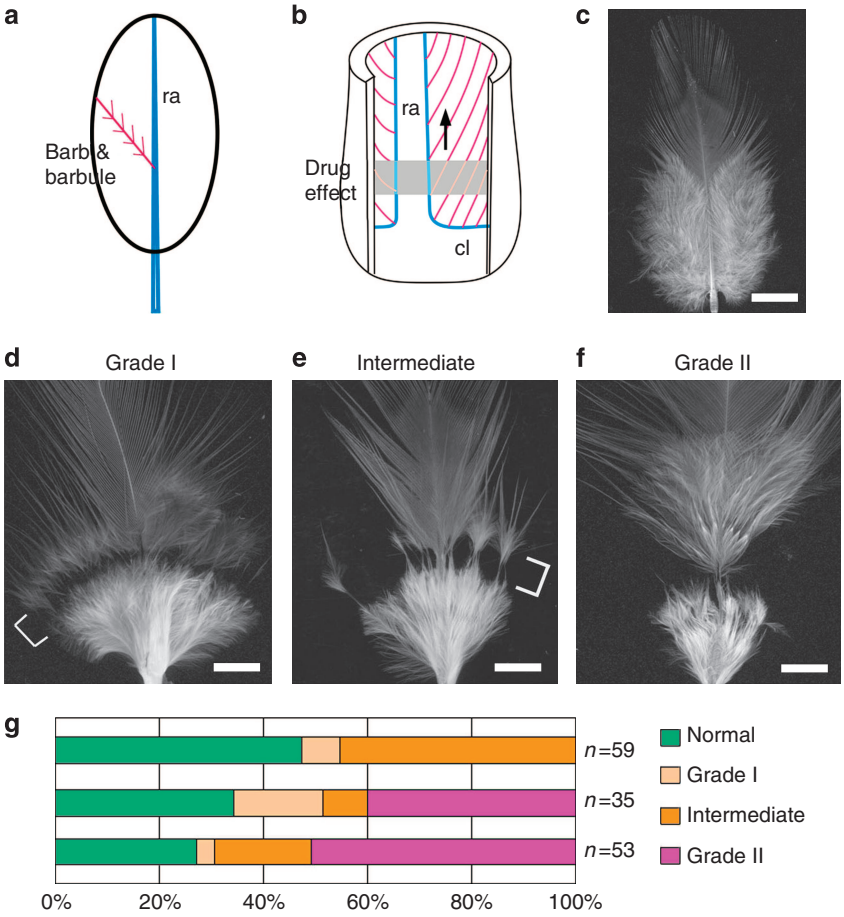

Figure 1. CYP disrupts feather formation. (**a** and **b**) Schematics showing the feather structure with barbs attach to the rachis, and barbules attach to barbs. The drug effect will be recorded in the morphology as the feather grows. ra, rachis; cl, collar where feather branching has not started. (**c**) A normal feather. (**d**–**f**) Representative examples of the feather morphology after CYP treatment. Showing the different grades of damage. The ''intermediate'' phenotype reveals that the barbs are broken down, which then leads to grade II defect. White bracket indicates the interval of drug perturbation. Bar = 1 cm. (**g**) Feather phenotypes showing the ''population variation'' among different birds and ''individual variation'' on the same bird (*n* is the number of feathers analyzed; each line represents one bird).

significant defects in feather formation (Figure 1d–f). These defects can be graded into two subtypes (Chen *et al.*, 2014). Grade I defects take a linear shape (Figure 1d): the feather barbs persist without barbules attached in short intervals. This specific type of feather defect is known as ''isochrones,'' meaning cells on this line are born at the same time: typically such a line forms in the feather when a bird experiences a short period of stress/starvation or ingests food with pigmentation-inducing properties (Lucas and Stettenheim, 1972; Prum and Williamson, 2001). Grade II defects are more severe. The lower edge of the defect remains a line, but the upper edge takes a ''V'' shape (Figure 1f). There are feathers showing an intermediate phenotype, which also reveals how grade II defect arises: some barbs remain but with no barbules attached in a short interval (Figure 1e). In all feathers, the central shafts (rachis) remain unaffected. Therefore, unlike mouse hair during CIA, feathers do not fall-out and finish their growth cycle normally.

Variation in the feather phenotype was seen both among different birds (population variation) and among different feathers in a single bird (individual variation; Figure 1g). About 25–50% of the feathers remained normal. These results suggest an effect independent of genetic backgrounds, and that the response of the feather to chemotherapy varies significantly, just as is typically seen with different hair follicles in mice and man (Yun and Kim 2007, Chon *et al.*, 2012, Paus *et al.*, 2013).

CYP treatment selectively disrupts the feather structure and induces intrafollicular apoptosis

Histological analysis revealed additional defects in feather formation after CYP treatment (Figure 2a and b). At day 1 post treatment (T1), the branching epithelium was already reduced. This became more obvious at day 2 (T2). Again, variation in response was noticed. In all cases, the rachis remained largely normal, whereas the branching barbs/barbules were reduced or disrupted in patterning, suggesting selective perturbation of the feather structure (Figure 2b). In some cases, we noticed disrupted feather branching similar to those observed after ionizing radiation treatment (Figure 2b and Supplementary Figure S1 online; Chen *et al.*, 2014), suggesting an impact of cytokine signaling. Indeed, an increased expression of TNFα and IFNγ in the feather follicles was noticed (Supplementary Figure S1 online). This is well in line with previous studies documenting altered cytokine production in human scalp hair follicles treated with a CYP derivative (Bodo *et al.*, 2007) and with the fact that anti-inflammatory hormone treatment significantly modulates the response of human hair follicles to chemotherapy-induced damage *in vitro* (Bodo *et al.*, 2009).

The reduced formation of feather branching could be due to reduced cell proliferation (see below) or increased cell apoptosis. Intrafollicular apoptosis is a major event during CIA (Lindner *et al.*, 1997, Botchkarev *et al.*, 2000; Sharov *et al.*, 2004; Bodo *et al.*, 2007; Paus *et al.*, 1994; 2013). Therefore, apoptosis in feather follicles was investigated with the DNA binding dye propidium iodide (PI). Apoptotic cells showed punctuated nuclei, which became obvious only in the branching area at T1 or T2 (Figure 2c). Per each barb there were 3–5 apoptotic cells, whereas no apoptotic cells were seen in the rachis. Increased apoptosis in the branching area, but very little in the rachis, was also demonstrated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Figure 3a and d).

Transmission electron microscopy (TEM) analysis remains the ''gold standard'' of cytomorphological evidence for cell apoptosis (Magerl *et al.*, 2001; Bodo *et al.*, 2007; Elmore, 2007). Again, this demonstrated normal euchromatin ultrastructure in the nuclei of rachis cells, whereas isolated cells in the branching area showed typical condensed heterochromatin, and/or apoptotic bodies (Figure 2d). This confirms that chemotherapy prominently induces apoptosis in distinct compartments of the feather follicle epithelium, thus documenting another parallel between the feather and hair follicle response to chemotherapy.

CYP treatment induces distinct molecular and cellular perturbations in the feather follicle

P53 activation and cell apoptosis are important in CIA (Botchkarev *et al.*, 2000; Sharov *et al.*, 2004; Paus *et al.*, 2013). We demonstrated the activation of P53 by CYP, which promoted nuclear enrichment of this protein both in the branching area and the rachis (Figure 3a). RT-PCR (reverse transcription-PCR) analysis showed increased expression of *p53/p21*, indicating activation of the P53 signaling pathway (Figure 3b and Supplementary Figure S4 online).

Other complex phenomena of drug-induced DNA damage and repair responses are also documented in the feather model. γ-H2AX immunostaining, which indicated chromatin modification after DNA damage, was increased (Figure 3a). Moreover, we found extensive nuclear localization of poly ADP-ribose polymerase (PARP) after CYP treatment. PARP is involved in DNA damage repair and serving as a substrate for caspase-3 during apoptosis (Wang *et al.*, 1997; Liu *et al.*, 2011). *Fas/Caspase-3* gene expression was increased, as expected (Figure 3b and Supplementary Figure S4 online). Together, feather follicles show typical responses to CYP treatment on multiple molecular levels that are expected from the hair research literature on CIA.

Selective inhibition of proliferation in the feather branching area suggests a role for Shh signaling

Next, we analyzed cell proliferation in the feather follicles. A significant reduction in proliferating cell nuclear antigen (PCNA) immunoreactivity in the branching area, but not the rachis, was observed (Figure 3a and c). To confirm a selective blockade of cell proliferation, we examined (5-Bromo-2-deoxyUridine) (BrdU) incorporation efficiency. Consistent with previous work (Yue *et al.*, 2005), most cells in the collar and the branching area (ramogenic zone) actively proliferated. After drug treatment, cells in the collar region still incorporated BrdU but not cells in the ramogenic zone. Similarly, cells in the rachis incorporated BrdU but not cells in the branching epithelium (Figure 4c). Therefore, CYP blocks cell proliferation only in the feather branching area. These results also explain why feathers do not fall out after drug treatment, because the rachises remain unaffected, and cells in the collar are able to replenish the feather branching post treatment.

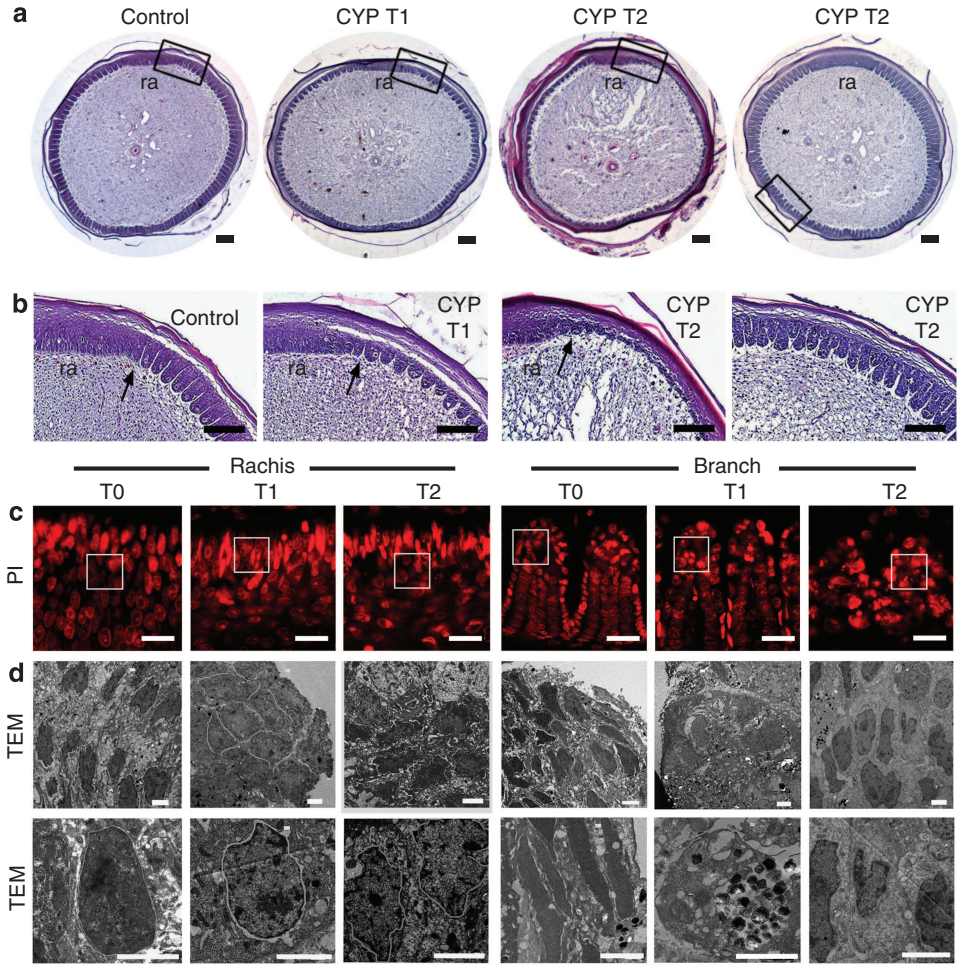

Figure 2. Analysis of CYP-induced feather defects. (**a** and **b**) H and E analysis showing the reduced feather branching but normal rachis (ra) after CYP treatment. The perturbed branching pattern is also shown. Two examples at day 2 post treatment (T2) are shown to illustrate the variation in response. T1, 1 day post treatment. Boxed areas in (**a**) are shown at a higher magnification in (**b**). (**c**) Propidium iodide (PI) staining and (**d**) TEM analysis of the feather epithelium. Notice the punctuated PI staining in T1 and T2 branching areas, euchromatin in rachis cell nucleus, apoptotic bodies or condensed nucleus, and membrane blebbing in branching areas after CYP treatment. Bar = 100 μm in (**a**, **b**), 20 μm in (**c**), and 2 μm in (**d**).

This distinct proliferation phenotype suggested a possible role of Shh signaling in mediating this damage. On one hand, Shh signaling is critically involved in the control of epithelial cell proliferation during feather/hair development and cycling (Ting-Berreth and Chuong, 1996; St-Jacques *et al.*, 1998; Chiang *et al.*, 1999; Botchkarev *et al.*, 2001; Oro and Higgins, 2003). On the other hand, *Shh* is not expressed in the rachis or collar region (Figure 4a) but only in the branching area where barb/barbule forms. Immunohistology confirmed reduced expression of *Shh* after CYP treatment (Figure 4b): Although Shh protein is normally expressed in the marginal plate of the feather epithelium (i.e. between barbs), it was undetectable 1 day post treatment. Reduced expression of *Shh* mRNA, together with that of the downstream target genes *Cdc25, Nmyc,* and *Cyclin D1* (Benazeraf *et al.*, 2006; Fan and Khavari, 1999; Mill *et al.*, 2005), was also confirmed (Figure 3b and Supplementary Figure S4 online).

Redistribution of the topobiologically important adhesion molecules, liver cell adhesion molecule (LCAM) and neural cell adhesion molecule (NCAM) (Chuong and Edelman, 1985; Lin *et al.*, 2013), was also observed (Figure 4b). Given that NCAM is not only important for feather development, but also involved in the control of murine hair follicle cycling (Combates *et al.*, 1997; Müller-Röver *et al.*, 1998) and that NCAM expression in the dermal papilla is greatly reduced in defective murine hair follicles (Panteleyev *et al.*, 1999), this reveals yet another parallel between the damage responses of feather and hair.

Downregulation of Shh signaling recapitulates the effect of CYP treatment in feather formation

To demonstrate that indeed downregulation of Shh signaling could contribute to CYP-induced defects, we directly manipulated Shh signaling in the feather follicle. Two independent methods were applied, and similar results were obtained.

(i) We delivered cyclopamine, a chemical inhibitor of Shh signaling, into the developing feather follicles using an agarose bead carrier (Yue *et al.*, 2006; Chen *et al.*, 2014). Cyclopamine

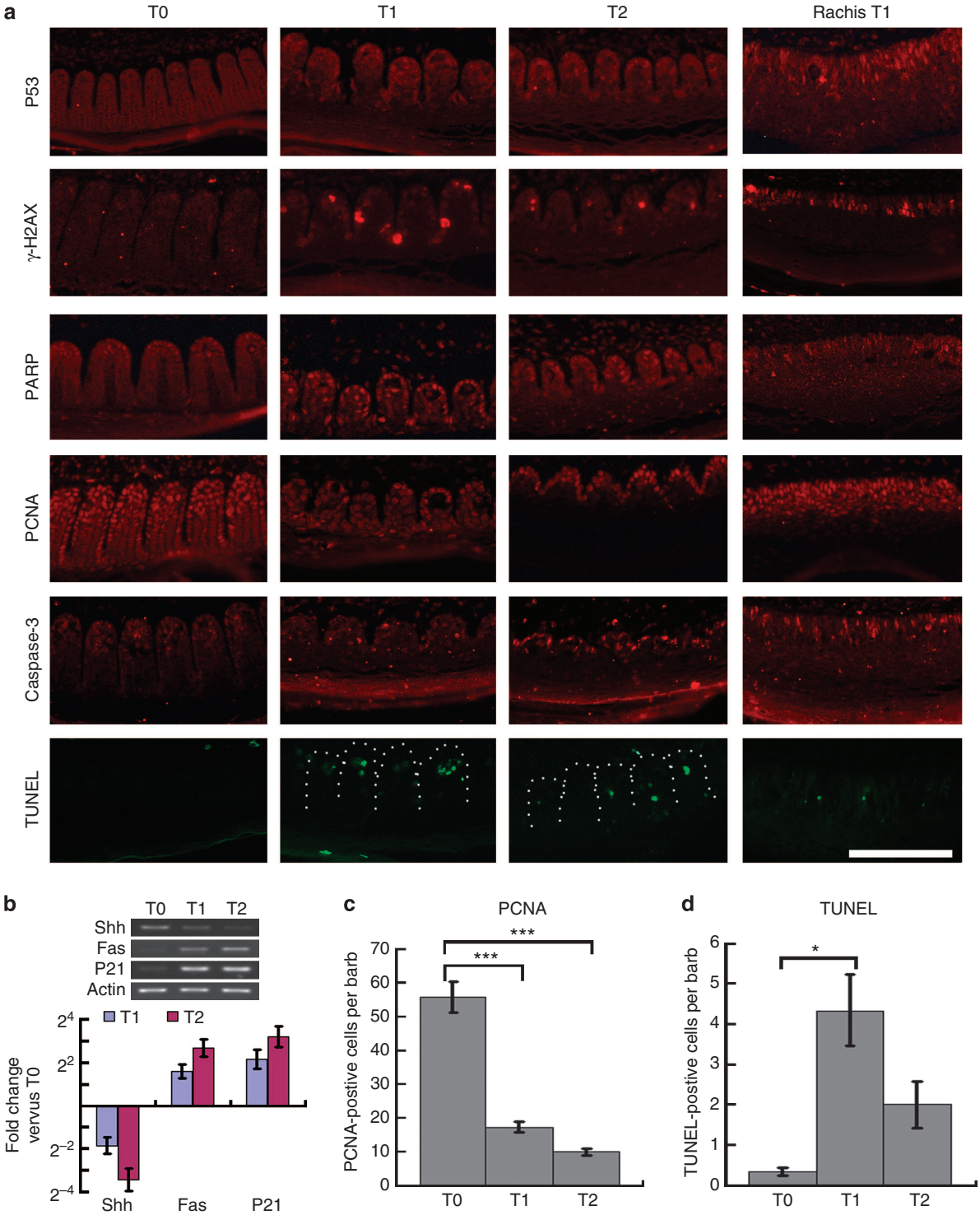

Figure 3. Molecular and cellular analysis in the feather follicle after CYP treatment. (**a**) Immunostaining of P53, γ-H2AX, PARP, PCNA, Caspase-3, and TUNEL staining in the feather follicle. Both the branching area (T1, T2) and the rachis (T1) are shown. CYP induces nuclear enrichment of P53 and activates γ-H2AX/PARP/Caspase-3 in both areas. Positive TUNEL staining is found mainly in the branching area, and downregulation of PCNA is induced only in the branching area. (**b**) RT-PCR and qPCR analysis showing increased expression of *p21*/*Fas* but decreased expression of *Shh*. Representative results from three independent experiments were shown, with each repeated three times. (**c** and **d**) Quantification of PCNA and TUNEL staining in feather branches (mean ± SD). Ten barbs were analyzed. *$P<0.05$; ***$P<0.001$. Bar = 100 μm. PCNA, proliferating cell nuclear antigen.

caused specific defects in feather formation (Figure 5a). In the perturbed area, the cell mass was significantly reduced with no branching formation. Hematoxylin and eosin (H and E) analysis confirmed this observation (Figure 5b). PCNA staining revealed decreased cell proliferation in the perturbed area, as compared with the neighboring branches (Figure 5c).

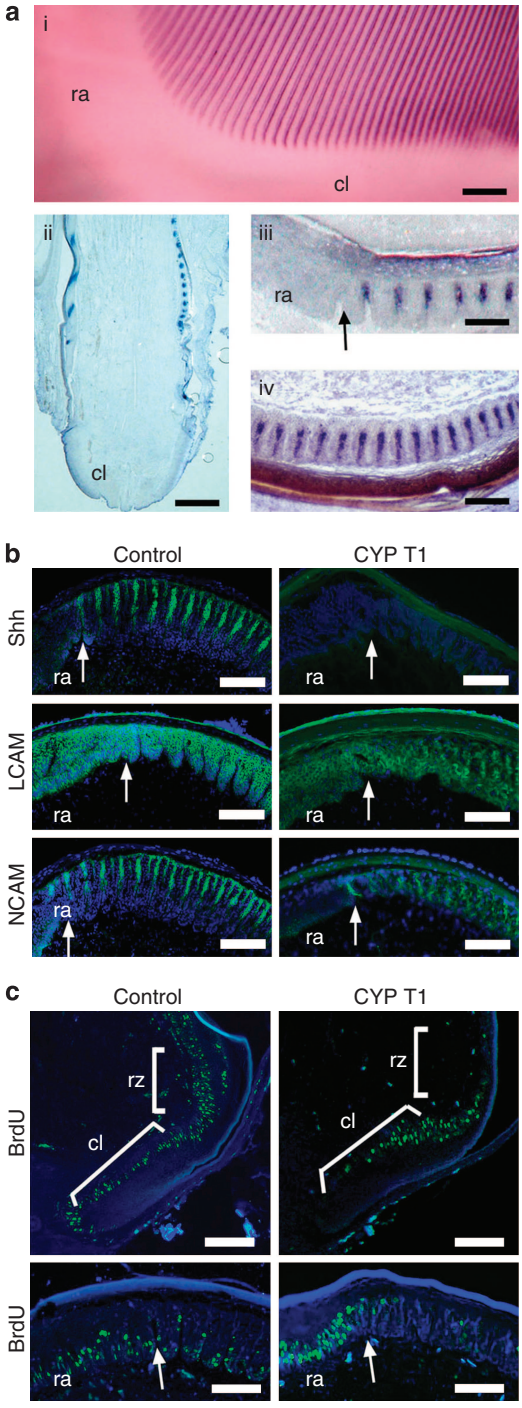

Figure 4. CYP treatment downregulates *Shh* gene expression and selectively blocks cell proliferation in the feather branching. (**a**) Expression of *Shh* mRNA in (i) whole mount, (ii) longitudinal section, and cross-section on the (iii) anterior and (iv) posterior side of the feather follicle. (**b**) Immunostaining and (**c**) BrdU staining of the feather follicles 1 day post treatment (T1). Bar = 100 μm. Arrows indicate the junction between the rachis and branches. cl, collar; ra, rachis; rz, ramogenic zone.

(ii) Using a recently developed method for lentiviral-mediated gene silencing in the feather follicle (Chu *et al.*, 2014), RNAi-*Shh* delivery was shown to reduce the cell mass, and no branching was formed in the perturbed area (Figure 5d and e). The efficiency of RNAi knockdown was about 60%, as monitored by quantitative PCR (qPCR) (Figure 5f). These results suggest that downregulation of Shh signaling causes defects in feather formation that are strikingly similar to those induced by CYP treatment.

Chemotherapeutic agents differ in their capability to downregulate Shh expression

To explore whether Shh signaling is universally perturbed by chemotherapeutic agents or only specific to CYP, we tested several other frontline drugs. Arabinofuranosyl Cytidine (Ara-C) and Dox are often associated with P53 activation and cell apoptosis. However, in our assays they did not disrupt feather formation. Although previous work suggested that Dox at 15 mg kg^{-1} i.p. injection induces alopecia in mice (Selleri *et al.*, 2005,2006; see below and Supplementary Figure S5 online), at this dose all chickens died. In contrast, 5-FU and taxol induced feather defects (Figure 6a). Overall, 5-FU induced defects in 90% of the feathers, whereas taxol was much less potent, with only 10% of the feathers showing grade I defect (Figure 6b).

Next, this selectivity in feather follicle damage was correlated with the impact on intrafollicular *Shh* expression. Ara-C- or Dox-treated feathers showed normal expression of Shh protein, whereas in 5-FU or taxol-treated feathers, Shh protein levels were significantly decreased (Figure 6c). Therefore, downregulation of *Shh* gene expression correlated well with morphological defects.

As 5-FU extensively damages feather formation, and remains an important chemotherapeutic agent in cancer therapy, we investigated its impact in the feather follicle further (Supplementary Figure S2 online). Similar to CYP, 5-FU also activated P53 signaling and increased *p53/p21* gene expression. γ-H2AX and PARP were activated, both in the branching area and the rachis. Downregulation of PCNA staining was found only in the branching area, but not the rachis, as expected. RT-PCR analysis confirmed downregulation of *Shh* gene expression, together with that of the downstream target genes (Supplementary Figure S2 and S4 online). However, 5-FU did not activate caspase-3 as strongly as CYP, which correlated with weaker TUNEL signals. PI staining and TEM analysis also revealed less apoptosis (Supplementary Figure S3 online). These observations in the feather model correspond well with the relatively lower incidence and milder phenotype of CIA in patients treated with 5-FU (Alley *et al.*, 2002, Chon *et al.*, 2012).

Key observations made in the feather model are reproduced in murine hair follicles *in vivo*

Finally, we tested directly whether any of these observations in the feather model is of relevance in mammalian skin biology, using the best-established mouse model of CIA (Paus *et al.*, 1994). This showed that the capacity of a tested chemotherapeutic drug to downregulate *Shh* gene expression correlated well with its capability to induce hair loss (Figure 6d and e). At the tested doses, 5-FU and Taxol induced significant hair loss and downregulated *Shh* gene expression, which is in significant contrast to Ara-C and Dox. On the other hand, the

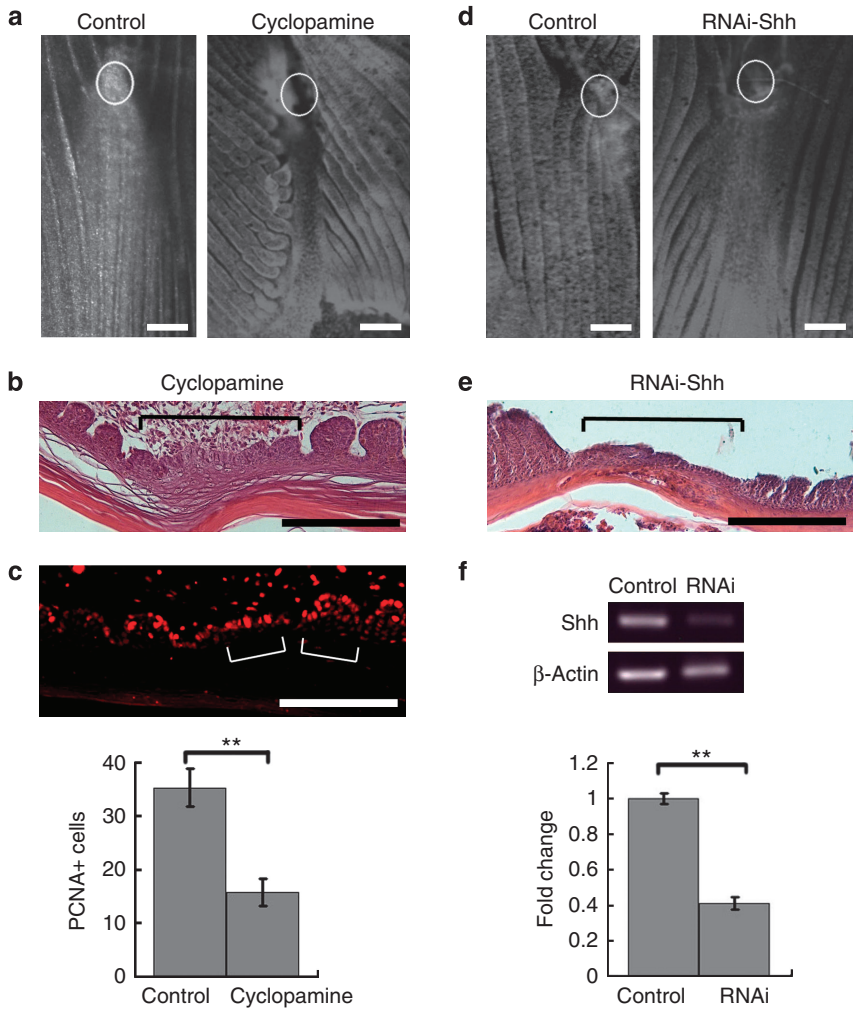

Figure 5. Downregulation of Shh signaling disrupts feather development. (**a**) Cyclopamine disrupts feather branching. Control samples were sham treated with PBS. Circles indicate the injection sites. (**b**) Hematoxylin and eosin (H and E) and (**c**) proliferating cell nuclear antigen (PCNA) staining showing reduced cell proliferation as compared with neighboring branches. Positively stained cells were counted (in the marked regions) and compared (mean ± SD). (**d**) Lentiviral-mediated RNAi silencing of *Shh* gene expression disrupts feather branching. A virus targeting a random sequence was used as control. Circles indicate the injection sites. Representative results of at least five repeats were shown. (**e**) H and E analysis showing RNAi-Shh treatment reduced the feather epithelium. (**f**) The efficiency of RNAi knockdown was monitored by RT-PCR and qPCR. $^{**}P<0.01$. Bar = 100 μm.

expression levels of *p21* and *Fas* do not correlate with the hair loss phenotype. At a higher dose, Dox treatment induced hair loss in mice and downregulated *Shh* gene expression (Supplementary Figure S5 online). These results suggest that our observations made in the feather system may hold true in murine hair follicles, and the key role of Shh signaling in chemotherapy-induced feather follicle damage also applies to mammalian skin appendages.

DISCUSSION

In this work, we show that despite its obvious distinctness, the experimentally well-tractable feather follicle harbors important lessons for hair follicle research and that the feather model can reveal signaling pathways that had previously escaped notice in CIA research (Chon *et al.*, 2012; Paus *et al.*, 2013). We reveal that chemotherapeutic agents selectively disrupt a defined portion of the adult feather structure and that downregulation of *Shh* gene expression correlates with this selectivity. As we show that some of these results are reproducible in murine hair follicles *in vivo*, our findings are likely to be relevant beyond avian biology and invite one to explore targeting the Shh pathway as a potential strategy for the future management of CIA.

Thus, despite the obvious differences between feather and hair (Paus and Cotsarelis, 1999; Yu *et al.*, 2004; Lin *et al.*, 2013), one can use the feather model to dissect general principles that govern the complex effects of chemotherapy reagents and other proliferation–/ apoptosis–inducing reagents on epithelial growth control and regeneration. Interestingly, the downregulation of *Shh* expression in chemotherapy-treated feather follicles identified here is faithfully reflected in murine anagen hair follicles treated with the same alopecia-inducing chemotherapeutic agents. This underscores that the feather follicle is indeed an excellent model for dissecting

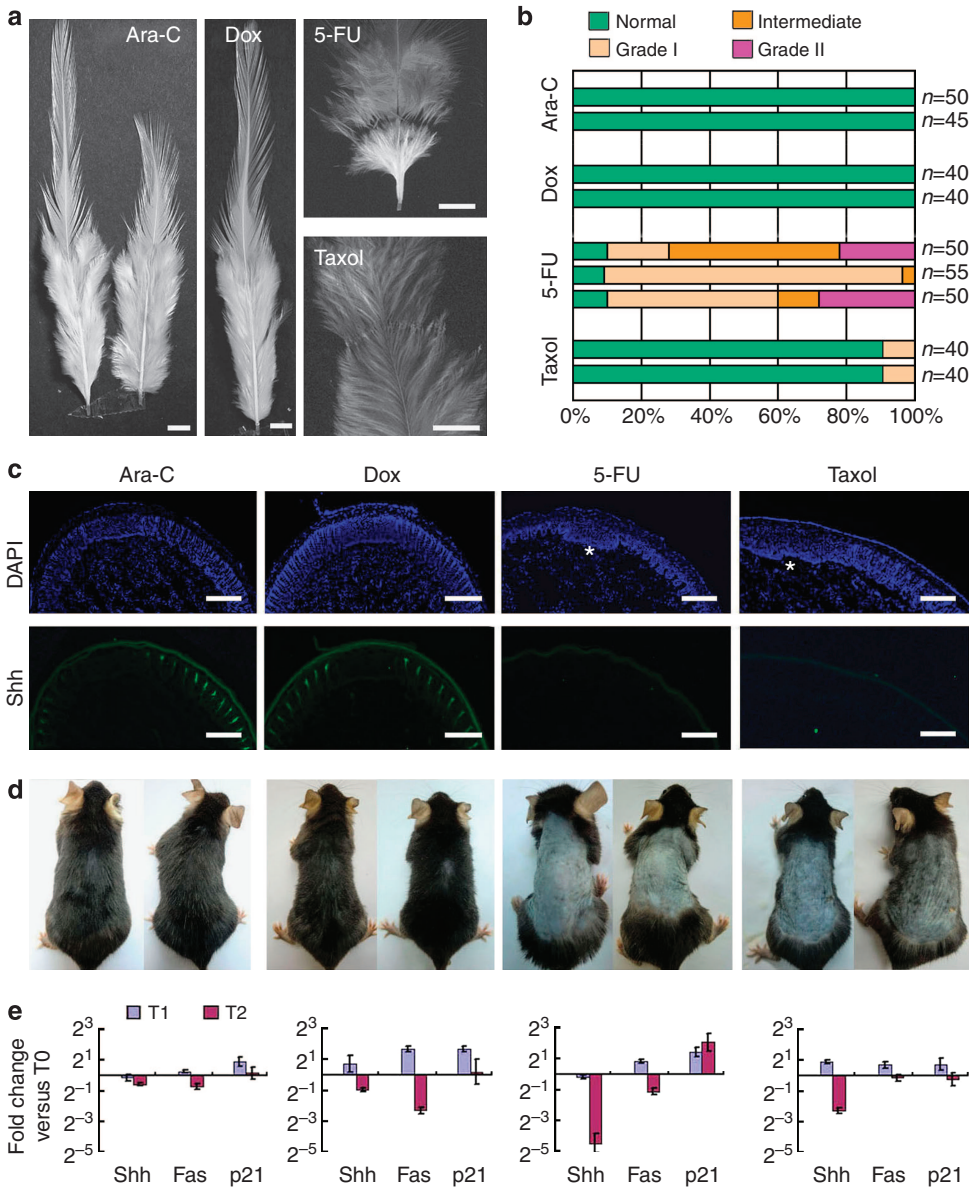

Figure 6. Downregulation of *Shh* expression correlates with the chemotherapeutic agent's capability to induce defects in feather formation and hair loss. (**a** and **b**) Morphological defects in feather formation are induced by 5-fluorouracil (5-FU) and Taxol but not by Ara-C and Dox. *n* is the number of feathers analyzed in each bird. Bar = 1 cm. (**c**) Immunostaining of Shh protein in the feather follicles (T2). Notice the normal rachises (marked by *) but reduced branches. Bar = 100 μm. (**d**) Ara-C ($n = 5$) and Dox ($n = 5$) do not cause hair loss, whereas 5-FU (6/8) and Taxol (2/6) can induce alopecia. (**e**) Quantification of gene expression in mouse skin. Relative to T0, *Shh* expression was significantly reduced by 5-FU or Taxol treatment at T2. Representative results from three independent experiments were shown, with each repeated three times.

evolutionarily conserved damage-response pathways of vertebrate skin appendages.

Shh signaling promotes hair regrowth in mice after CIA (Sato *et al.*, 2001), and Shh agonists are hair-growth promoting (Paladini *et al.*, 2005). Yet, the role of Shh signaling in mediating chemotherapy-induced tissue damage as such had not been investigated or even considered in CIA pathobiology research (Paus *et al.*, 2013). Obviously, before the potential clinical relevance of our findings in feather and murine hair follicles can be judged, the role of Shh signaling should be further investigated in chemotherapy-damaged human scalp hair follicles, using established *in vitro* (Bodo *et al.*, 2007) and *in vivo* systems (Paus *et al.*, 2013) (see Supplementary discussion online on chemotherapy dosage considerations and interfollicular variations of the feather response to chemotherapy).

The regulation of *Shh* gene expression in hair and feather development remains incompletely understood. It is known that ectodysplasin A/ectodysplasin A receptor and Wnt signaling act upstream of *Shh* expression and that TNFα activates *Shh* expression in embryonic skin (Schmidt-Ullrich *et al.*, 2006; Zhang *et al.*, 2009). However, it is unclear whether

there are hitherto unknown regulatory mechanisms under pathological conditions that come along with the metabolic cell stress and the generation of reactive oxygen species induced by chemotherapeutic agents. Previous work suggested antioxidants could ameliorate CIA (reviewed in Wang *et al.*, 2006; Trueb, 2009), and our preliminary results suggest that reactive oxygen species may downregulate *Shh* gene expression (not shown). Downregulation of *Shh* gene expression is a rather quick response by CYP treatment—i.e. within 24 hours *Shh* mRNA was reduced to one fourth of the original level in the feather follicle. Morphologically, this coincides with or precedes obvious tissue damage both in feather (Figure 2) and hair (Supplementary Figure S6 online). Therefore, an intraepithelial mechanism appears to be in place that directly downregulates *Shh* gene expression. Subsequent studies are required to clarify the upstream signaling cascades.

In summary, we propose an additional layer of complexity in the pathobiology of chemotherapy-induced tissue damage by introducing Shh–regulated proliferation control in distinct epithelial cells. This pathomechanism may be targeted therapeutically and likely requires an approach that is distinct from the commonly discussed anti-apoptosis strategies for managing CIA (Paus *et al.*, 2013). Conceivably, this Shh-dependent pathomechanism may extend to the chemotherapy response of other tissues/organs. Furthermore, as we show here that not all proliferating cells in the feather follicle will respond equally to anti-proliferative, apoptosis-promoting chemotherapeutic agents, one wonders if a similarly distinct, Shh-associated response also occurs in cancer cells during chemotherapy.

MATERIALS AND METHODS

Experimental animals and procedures

Six months old male chickens (*Gallus gallus domesticus*) were bought from a local farm maintained as an outbred stock. Male C57BL/6 mice were purchased from Shanghai SLAC animal facility center. All experimental protocols were approved by Fuzhou University Experimental Animal Ethics Board. Active feather growth was induced by a plucking-regeneration protocol (Chen *et al.*, 2014), and active hair growth was induced as described previously (Paus *et al.*, 1994). The doses used were as follows: CYP (Sigma, Shanghai, China) $150\,mg\,kg^{-1}$, Ara-C (Sigma) $50\,mg\,kg^{-1}$, Doxorubicin (BBI, Shanghai, China) $10\,mg\,kg^{-1}$, 5-FU (Meryer, Shanghai, China) $150\,mg\,kg^{-1}$, and Taxol (BBI) $100\,mg\,kg^{-1}$. Feathers were each individually marked and followed, and collected at designated times for analysis, or left for another 3 weeks to finish the growth cycle. For gene expression analysis in mice, whole skin samples (5×5 mm) were collected at designated times.

Histology, immunostaining, and TUNEL staining

HE staining, immunostaining, TUNEL staining, and *in situ* hybridizations were processed as described (Chen *et al.*, 2014). Briefly, feather follicles were fixed by 4% paraformaldehyde at 4 °C overnight and processed for paraffin section. For PI staining, sections were stained with $1\,\mu g ml^{-1}$ PI in PBS for 30 min and mounted. For BrdU incorporation, animals were i.p. injected with $50\,mg\,kg^{-1}$ BrdU (Sigma) and samples collected 1 hour later. The following antibodies were used: BrdU, Shh, LCAM, NCAM (Hybridoma Bank, Iowa City, IA), P53, PARP, PCNA, Caspase-3 (Santa Cruz, Dallas, TX), and γ-H2AX (Abcam, Cambridge, UK).

TEM analysis

Feather samples were dissected and processed for ultrathin section using the standard protocol. Briefly, samples were fixed with primary fixative (0.2% tannic acid, 3% Tousimis glutaraldehyde in MOPS buffer) and secondary fixative (1% OsO4 in water), stained with 2% Ur-acetate, and mounted in resin. Ultrathin sections of 90 nm were collected and examined under an FEI Tecnai G2 Spirit TEM.

RT-PCR and qPCR analysis

An average of four feather follicles were collected from the chicken at designated times, and total RNAs extracted using the Trizol reagent (Shanghai Sangon, Shanghai, China). Equal loading was monitored by endogenous β-actin gene expression. For qPCR, each sample was analyzed in triplicate using SYBR green (CWBIO, Beijing, China) and a LightCycler480 real-time PCR machine (Roche, Basel, Switzerland). Data are normalized to β-actin.

Lentivirus production and manipulation of feather growth *in vivo*

Lentivirus production and monitoring of RNAi efficiency were described previously (Chu *et al.*, 2014). Sequences targeted: Shh (5′-ttctcttggtgggcttca-3′), and a random sequence was used as control (5′-agatacgacagaggacact-3′). The efficiency of RNAi knockdown was monitored in 293 T cells where a full-length chicken *Shh* gene was stably transfected. Manipulation of feather growth by chemicals or RNAi was described previously (Chen *et al.*, 2014). Cyclopamine was used at a final concentration of 500 μM.

Statistics

Data were expressed as mean ± SD. The statistical difference between two groups was determined by the two-tailed *t*-test, and the *P*-value was calculated.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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