Immunolocalization of Enzymes, Binding Proteins, and Receptors Sufficient for Retinoic Acid Synthesis and Signaling During the Hair Cycle

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Retinoic acid (RA) is essential for maintenance of most epithelial tissues. One RA biosynthesis pathway consists of cellular retinol-binding protein (Crbp), retinol dehydrogenase (Dhrs9/eRoldh), retinal dehydrogenase 1-3 (Aldh1a1-3), and cellular RA-binding protein 2 (Crabp2). Previously, we localized Aldh1a2 and Aldh1a3 to both epithelial and mesenchymal cells within the hair follicle throughout the hair cycle. This study expands that observation by examining the complete pathway of RA biosynthesis and signaling via RA receptors α, β, and γ by immunohistochemistry in C57BL/6J mice wax-stripped to initiate and synchronize the cycle. This pathway of RA biosynthesis and signaling localized to the majority of layers of the hair follicle, sebaceous gland, and interfollicular epidermis in a hair cycle-dependent manner, suggesting that RA biosynthesis within the hair follicle is regulated in both a spatial and temporal manner. This localization pattern also revealed insights into epithelial–mesenchymal interactions and differentiation state differences within the RA biosynthesis and signaling pathway, as well as novel observations on nuclear *versus* cytoplasmic localization of Crabp2 and RA receptors. This complex pattern of RA biosynthesis and signaling identified by immunolocalization suggests that endogenous RA regulates specific aspects of hair follicle growth, differentiation, and cycling.

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INTRODUCTION

Vitamin A and its derivatives (retinoids) are essential for the development and maintenance of multiple tissues, including skin and hair (Wolbach and Howe, 1925; Frazier and Hu, 1931). Retinoic acid (RA) is the active form of vitamin A. The synthesis of RA from circulating retinol occurs by the action of two enzyme families (Napoli, 1999). Retinol dehydrogenases (Roldhs) convert retinol into retinal, whereas retinal dehydrogenases (Raldh/Aldh1a) convert retinal into RA. There are several classes of Roldhs but only the short-chain dehydrogenases/reductases (SDR family), localized on the endoplasmic reticulum, recognize and oxidize retinol bound to cellular retinol-binding protein (Crbp, contested gene name *Rbp1*) (Napoli, 1999). Because of this feature, these Roldhs are argued to be the physiological enzymes that produce RA for hormonal signaling, although other Roldhs may also contribute. The SDR family member dehydrogenase reductase 9 (Dhrs9), identified in this laboratory as eRoldh (Rexer and Ong, 2002) (also known as hRDH-TBE, hRoDH-E2, (Chetyrkin *et al.*, 2001; Soref *et al.*, 2001; Markova *et al.*, 2003) is present in the majority of vitamin A-sensitive epithelial cells (Everts *et al.*, 2005). Currently, there are three known Raldhs (gene names *Aldh1a1–3*) found in mice, rat, and human that convert retinal into RA. *Aldh1a2*^{tm1Ipc}-null mice die *in utero* due to defects in heart development (Niederreither *et al.*, 1999) and *Aldh1a3*$^{tm1.1Pcn}$-null mice die within 10 hours of birth due to defects in nasal development (Dupe *et al.*, 2003), implying that they are essential for RA synthesis. But *Aldh1a1*^{tm1Gdu}-null mice have no apparent phenotype (Fan *et al.*, 2003). Although analysis of *Aldh1a1*, *Aldh1a2*, and *Aldh1a3* single-, double-, and triple-null mice revealed a redundant role for *Aldh1a1* in eye morphogenesis (Molotkov *et al.*, 2006), Aldh1a1 may also be involved in the catabolism of excess retinol (Fan *et al.*, 2003; Molotkov and Duester, 2003). Two binding proteins, Crbp and cellular RA-binding protein type II (Crabp2), are also closely associated with RA synthesis (Bucco *et al.*, 1997; Napoli, 1999). Crbp acts to chaperone retinol and retinal to retinoid metabolizing enzymes (Ong *et al.*, 1988; Napoli, 1999), whereas Crabp2 carries RA to the nucleus, where it shuttles RA to RA receptors (Rars) and increases transcriptional efficiency (Dong *et al.*, 1999; Budhu and Noy, 2002; Sessler and Noy, 2005).

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Abbreviations: Aldh1a1, 2, 3, retinal dehydrogenase1, 2, 3; Crabp1, cellular retinoic acid-binding protein I; Crabp2, cellular retinoic acid-binding protein 2; Crbp, cellular retinol binding protein; Dhrs9, dehydrogenase reductase (SDR family) member 9; IR, immunoreactivity; RA, retinoic acid; Raldhs, retinal dehydrogenases; Rara, b, c, retinoic acid-receptor α, β, γ; Roldhs, retinol dehydrogenases; Wnt, wingless related mouse mammary tumor virus

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Three Rars have been described (Rar α (Rara), Rar β ((Rarb), and Rar γ (Rarg)), which bind DNA and regulate transcription of specific genes in an RA-dependent manner. The binding of Crabp2 and Rar has only been proven for Rara, but may also occur with Rarb and/or Rarg. In contrast to Crabp2, Crabp1 is not directed to the nucleus on RA binding and does not bind Rars (Dong *et al.*, 1999; Budhu and Noy, 2002). Crabp1 may be involved in RA catabolism, but this is still unclear (Boylan and Gudas, 1992; Chen *et al.*, 2003). Therefore, it can be assumed that expression of these binding proteins, enzymes, and receptors indicates sites of RA synthesis and action. This assumption is supported by similar localization patterns between defects seen in vitamin A-deficient rat embryos and *Aldh1a2*- and *Aldh1a3*-null mice with immunohistochemical and *in situ* hybridization localization patterns of enzymes and binding proteins involved in RA synthesis (Bavik *et al.*, 1997; Niederreither *et al.*, 1999; Blentic *et al.*, 2003; Dupe *et al.*, 2003). The expression pattern of the complete system for RA synthesis and signaling in the hair follicle and sebaceous gland throughout the hair cycle has not been defined. Only individual components of this system were examined by different laboratories and no integrated understanding of all components has yet been attempted to our knowledge (Siegenthaler *et al.*, 1984; Billoni *et al.*, 1997; Reichrath *et al.*, 1997; Markova *et al.*, 2003). *Crbp*, *Aldh1a1*, *Aldh1a3*, and *Crabp2* were found in a screen of genes altered by the hair cycle, but a detailed analysis of their location was not performed (Lin *et al.*, 2004). Similar analysis of the complete system for RA synthesis in numerous rat epithelial tissues implicated both epithelial and mesenchymal cells in the synthesis of RA, which suggests a novel requirement for the movement of retinal between cell types (Li *et al.*, 2004; Everts *et al.*, 2005).

The hair cycle proceeds through four main stages: growth (anagen), regression (catagen), rest (telogen), and release (exogen). Signaling between epithelial and mesenchymal cells in the stem cell containing bulge and transiently proliferating cell containing bulb regions regulates this cycle. Previously, we reported that Aldh1a2 was expressed in the bulge region during anagen and early catagen, whereas Aldh1a3 expression alternates between the mesenchymal dermal papilla and epithelial precortex in the bulb region during mid-late anagen (Everts *et al.*, 2004). This study extends that observation and characterizes the expression of the complete system for RA biosynthesis and signaling including Crbp, Dhrs9, Aldh1a1, Aldh1a2, Aldh1a3, Crabp2, Rara, Rarb, and Rarg during the hair cycle in wax-stripped C57BL/6J mice. This complete system was expressed in the majority of layers of the hair follicle, sebaceous gland, and interfollicular epidermis in a hair cycle-dependent manner. Components were not always all in the same cell layer suggesting epithelial–mesenchymal interactions are important in this tissue as was seen for other epithelial tissues. In addition, in some sites expression of the RA synthesis and signaling pathway flowed through different stages of differentiation, with the most differentiated cells expressing the final proteins in this pathway – nuclear Crabp2 and Rars.

RESULTS

This study characterized the cell-specific location of all known components necessary for RA biosynthesis and signaling in the depilation-induced hair follicle both to obtain predictions of where endogenous RA fits into the signaling network that regulates the hair cycle, as well as to better understand the physiological pathway of RA biosynthesis and signaling. As RA synthesis cannot be measured directly, we are assuming that presence of enzymes, proteins, and receptors equates to RA synthesis and signaling based on similar assumptions made in the embryo during development (see Introduction). In addition, RA is diffusible and can act in either the cell where it is produced (autocrine) or in a neighboring cell (paracrine).

RA biosynthesis enzymes, binding proteins, and receptors localized to the stem cell niche

The bulge has been shown to contain stem cells that produce cells for the cycling lower half of the hair follicle (Cotsarelis, 2006). In telogen samples collected immediately after depilation (day 0), the RA biosynthesis and signaling pathway was weak or absent, except for localization of Rarb within the bulge and Rara just outside the bulge (Tables S1 and S2). In the second cycle of telogen (21–30 days after depilation) immunoreactivity (IR) of the RA biosynthesis and signaling system within the bulge was more intense, especially for Crabp2. During anagen I, Dhrs9, Aldh1a2, Rara, Rarb, and Rarg localized within the bulge and Rara and Crabp2 were localized to cells just outside the bulge (Tables S1 and S2). IR for RA synthesis proteins was greater during anagen II with the additional weak appearance of Aldh1a1 (Figure 1a, d, g, m, and p, arrowhead; 1j and m, long arrow). Rarb was localized to the cytoplasm, whereas Crabp2, Rara, and Rarg were both cytoplasmic and nuclear-localized. This localization pattern of RA synthesis and signaling in the stem cell niche remained relatively constant throughout anagen V, except Aldh1a3 appeared both within the bulge and, with greater IR, in the cells outside during anagen IIIb–V (Table S1). During anagen VI/catagen I the IRs of Dhrs9, Aldh1a1, Aldh1a2, Aldh1a3, Rara, Rarb, and Rarg were reduced, whereas Crabp2 localization switched from outside to within the outer edge of the bulge (Table S1, Figure 3a, d, e, i, l, m, p, q and t, arrowhead). Crbp IR within the bulge increased during anagen V then faded through catagen. Dhrs9 and Rarb localized to the bulge through catagen IV and then faded. Aldh1a1 and Aldh1a3 were not seen in the bulge beyond anagen V, whereas Aldh1a2, Crabp2, Rara, and Rarg remained in the bulge throughout the hair cycle.

RA biosynthesis enzymes, binding proteins, and receptors localized to both matrix and dermal papilla cells, as well as their precursor cells

The bulb is formed in early anagen from epithelial cells that proliferate and migrate from the bulge stem cells, which eventually enclose the dermal papilla (Cotsarelis, 2006). Once in the bulb, these cells are called matrix cells and are argued to be transiently amplifying cells. During anagen

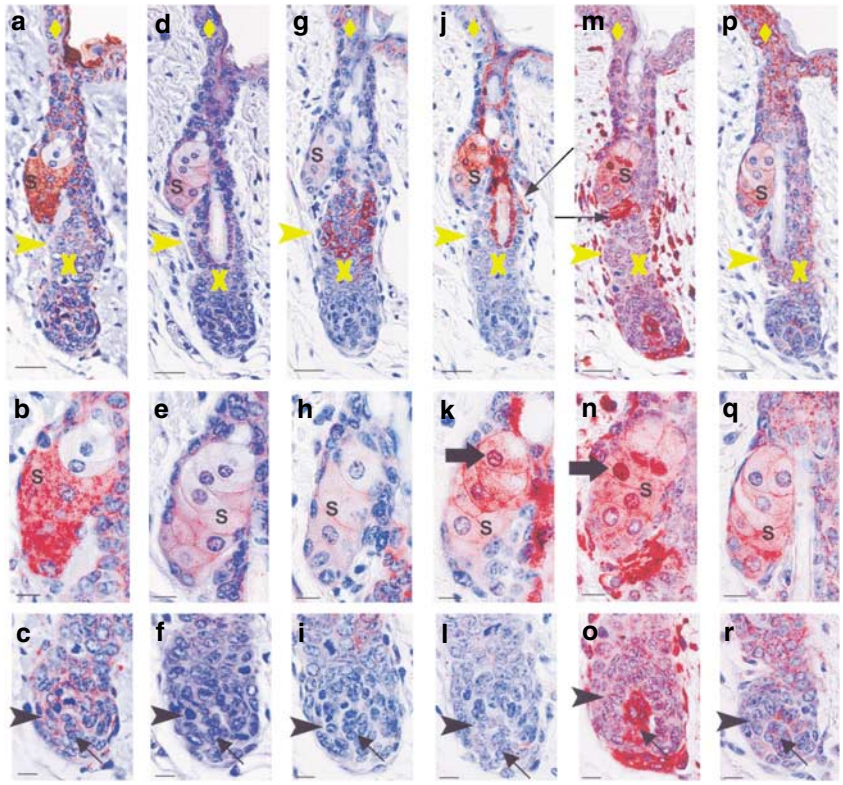

Figure 1. Localization of RA biosynthesis and signaling pathway in anagen II hair follicles. Localization of (**a–c**) Dhrs9, (**d–f**) Aldh1a1, (**g–i**) Aldh1a2, (**j–l**) Crabp2, (**m–o**) Rara, and (**p–r**) Rarb in anagen II hair follicles from C57BL/6J mice that were wax-stripped to induce anagen. For **a**, **d**, **g**, **j**, **m**, and **p,** bar = 25.2 μM, for **b**, **c**, **e**, **f**, **h, i**, **k**, **l**, **n, o**, **q**, and **r,** bar = 10.1 μM. Yellow arrowhead, bulge; yellow diamond, epidermis; yellow X, proliferating keratinocytes; S, sebocytes; long arrow, cells outside bulge; block arrow, nuclear localization; black arrowhead, matrix; short arrow, dermal papilla.

the mesenchymal connective tissue sheath that surrounds the bottom portion of the hair follicle proliferates and migrates into the dermal papilla (Tobin *et al.*, 2003). Rara localized to the connective tissue sheath surrounding the lower hair follicle, which peaked during anagen IIIa, dropped during anagen VI/catagen I, and remained weak through catagen (Figure 1m–o, data not shown). Rara and Rarg localized in the dermal papilla throughout the hair cycle with a slight drop during anagen VI/catagen I (Table S2, Figure 2d, h, l, and p, arrow, Figure 3q and r). Aldh1a3 appeared in the dermal papilla in anagen IIIb, peaked during anagen IV, dropped during anagen VI/catagen I, then was no longer seen (Table S2, Figure 2b, f, j, and n, arrow; Figure 3i and j). Dhrs9 was seen in the dermal papilla from anagen I–catagen IV, peaked during anagen I, II, and IV, then faded during catagen V (Table S2, Figure 1a and c, Figure 2a, e, i and m, short arrow). Crabp2 IR appeared in the dermal papilla during anagen IIIa, peaked during anagen IV, dropped during anagen V, was weak during catagen I–IV, then faded (S2, Figure 2c, g, k, and o, arrow; Figure 3m and n). Crbp, Dhrs9, Aldh1a2, Rara, Rarb, and Rarg were seen in the proliferating and migrating keratinocytes during early anagen (S1, Figure 1a, g, m, and p, yellow x). Crabp2 localized to the nucleus of a few of these cells (Table S1), but only Dhrs9, Rara, Rarb, and Rarg IR were seen in these cells once they reside in the bulb (matrix cells), beginning in anagen II and remained present throughout anagen (Table S2, Figure 1a, c, m, o, p, and r, arrowhead, Figure 2a–p, arrowhead).

RA biosynthesis enzymes, binding proteins, and receptors localized to the outer root sheath, companion layer, inner root sheath, and hair fiber

Dhrs9, Rarb, and Rarg IR were seen in the permanent outer root sheath (isthmus and infundibular) throughout the hair cycle, but Crbp, Aldh1a2, Crabp2, and Rara IR were more variable (Table S1, Figure 1a, d, g, j, m, and p). As the lower outer root sheath forms, Dhrs9, Aldh1a2, and Rarb appeared in a gradient with the greatest IR occurring near the bulge and reduced IR downward as was previously reported for Aldh1a2 (Everts *et al.*, 2004; Table S1). This pattern remained throughout the lifespan of these cells, but IR was less during catagen. But Crabp2 localization within the outer root sheath was weak or absent from anagen IIIa–IV, increased during anagen V, became constant and strong during anagen VI/catagen I–III, formed a gradient upward during catagen IV and then faded (Table S1, Figure 3m–p). Crbp appeared in the outer root sheath in a similar pattern as Crabp2, but increased during anagen IV and remained strong through catagen VI (Table S1, Figure 3a–d). Rara and Rarg were also present in the outer root sheath, but localization appeared more constant (Table S1, Figure 3q–t). Rara also localized to the connective tissue sheath surrounding the lower hair follicle

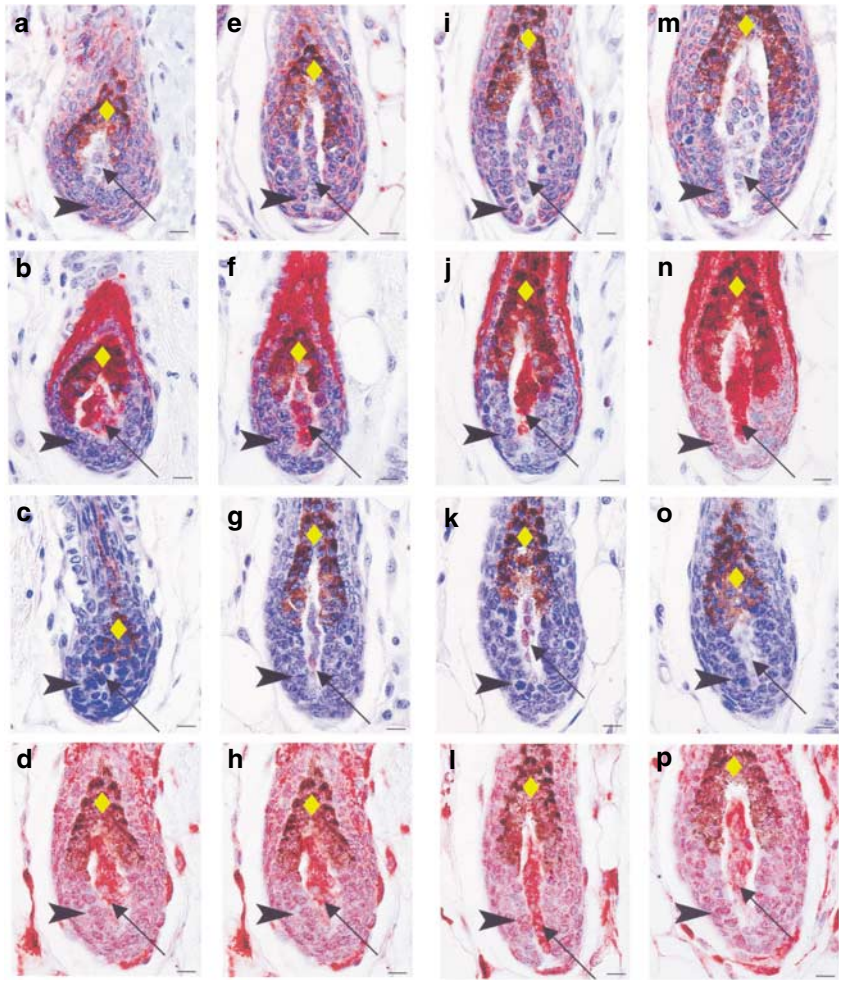

Figure 2. Localization of RA biosynthesis and signaling pathway in the bulb during anagen. Expression of (**a**, **e**, **i**, and **m**) Dhrs9, (**b**, **f**, **j**, and **n**) Aldh1a3, (**c**, **g**, **k**, and **o**) Crabp2, and (**d**, **h**, **l**, and **p**) Rara in (**a–d**) anagen IIIb, (**e–h**) anagen IIIc, (**i–l**) anagen IV, and (**m–p**) anagen V hair follicles from C57BL/6J mice that were wax-stripped to induce anagen. Bar = 10.1 μM. Yellow diamond, premedulla/precortex; black arrowhead, matrix; black arrow, dermal papilla.

with greater IR than the outer root sheath during anagen I–V (Figure 1m–o, data not shown).

The companion layer was one of the few sites to contain both Aldh1a2 and Aldh1a3. Aldh1a3 and Rara localized to the companion layer as soon as this structure developed in anagen IIIa and remained in a gradient of strong to weaker IR as this layer differentiated upward through catagen V (Table S3, Figure 3i–l, q–t, short arrow). Aldh1a3 and Rara IR decreased during catagen VI–VIII and were localized more in the isthmus. By contrast, Aldh1a2 was seen to various degrees during anagen IIIc–catagen VIII with a gradient of weak to stronger IR as this layer differentiated upward (Table S3). Rarb was seen in the companion layer during anagen IIIb–V, and catagen III in a gradient of weak to strong IR, but this gradient was reversed and more variable during anagen VI/catagen I–II and catagen IV–VIII. Dhrs9 and Crabp2 localized to the companion layer with a less apparent gradient throughout the hair cycle when this layer is present (Table S3, Figure 3e–h, m–p, short arrow). Crbp was seen in the companion layer more variably peaking during anagen VI/catagen I (Table S3, Figure 3a–d, short arrow).

Dhrs9 and Aldh1a3 appeared in the inner root sheath cone as it formed during anagen IIIa and remained strong through anagen VI/catagen I, then reduced IR through catagen VIII (Table S2, Figure 3e–h, i–l, yellow x). Crbp and Crabp2 were seen in the inner root sheath from anagen IIIc–catagen IV, peaking during anagen VI/catagen I (Table S2, Figure 3a–d, m–p, yellow x). Rara and Rarg localized to the inner root sheath from anagen IIIa–catagen V (Table S2, Figure 3q–t, yellow x). This system for RA synthesis and signaling showed a gradient with the strongest IR near the bulb and reduced IR as these cells differentiated and moved upward. Aldh1a3 IR was greater in the outer Henle's layer throughout anagen (Figure 3k, yellow arrow), whereas Crabp2 IR appeared strongest in the inner root sheath cuticle during anagen VI/catagen I only (Figure 3o, fat arrow).

Aldh1a3 localized to the hair fiber with a gradient of strong IR occurring in the precortex/premedulla and reduced IR as these cells differentiate and move upward throughout anagen (Table S2, Figure 2b, f, j, and n and Figure 3i–l, yellow diamond). Aldh1a3 IR was stronger in the premedulla and medulla than precortex and cortex. Dhrs9 localized variably to

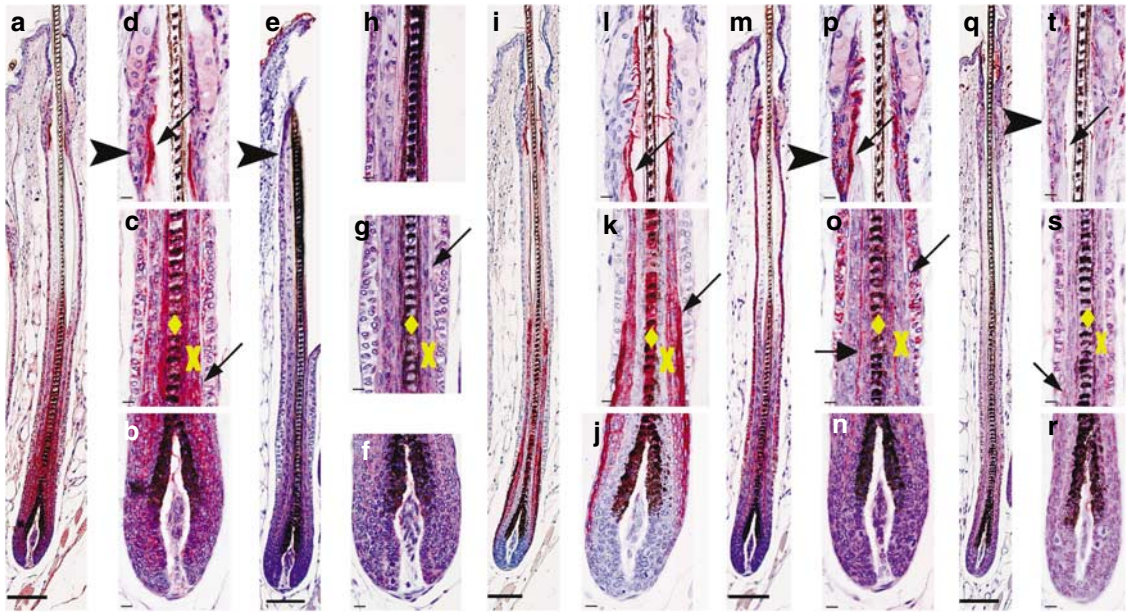

Figure 3. Localization of RA biosynthesis and signaling pathway in anagen VI/Catagen I hair follicles. Expression of (**a–d**) Crbp, (**e–h**) Dhrs9, (**i–l**) Aldh1a3, (**m–p**) Crabp2, and (**q–t**) Rara in anagen VI/catagen I hair follicles from C57BL/6J mice that were wax-stripped to induce anagen. For **a**, **e**, **i**, **m**, and **q,** bar = 45.8 μM, for **b–d**, **f–h**, **j–l**, **n–p**, and **r–t**, bar = 10.1 μM. Black arrowhead, bulge; yellow diamond, hair fiber; yellow X, IRS; skinny arrow, CL; thick arrow, IRS cuticle.

the precortex and premedulla during anagen IIIa–IV, with a peak during anagen IIIb, and was seen in the medulla during anagen IIIb and anagen VI/catagen I–III (Table S2, Figures 2a, e, i and m, and 3e–h, yellow diamond). Rara and Rarg localized to the premedulla/precotex and hair fiber in a gradient similar to Aldh1a3 throughout anagen (Table S2, Figure 2d, h, l and p and Figure 3q–t, yellow diamond). Crbp localized to the precortex during anagen V and then expanded IR to also include the premedulla and hair fiber during anagen VI/catagen I (Table S2, Figure 3a–d, yellow diamond). Crabp2 IR was faint or negative in the precortex/premedulla throughout the hair cycle, but weak and strong in the more differentiated medulla during anagen V and anagen VI/catagen I, respectively (Table S2, Figures 2c, g, k, o, and 3m–p, yellow diamond).

RA biosynthesis enzymes, binding proteins, and receptors localized to the sebaceous gland primarily during early-mid anagen

Little is known about changes within the sebaceous gland during the hair cycle, but by observation the size of this gland changes and is the largest during early anagen (I–IIIa) and smallest during late anagen and early catagen (Everts HB, unpublished observation; Paus and Foitzik, 2004). Although sebaceous gland size does not predict activity (Thody and Shuster, 1989), different types of cells (undifferentiated, maturing, mature) could only be distinguished in the larger sebaceous glands. Undifferentiated cells occur on the entire periphery of the sebaceous gland but only the ones on the bottom are thought to differentiate as they migrate upward (maturing). Mature sebocytes become completely filled with lipid, die, rupture, and release their contents into the hair canal. Localization of the RA biosynthesis and signaling pathway varied in the sebaceous gland during the hair cycle. Throughout the hair cycle, Dhrs9 IR was strong in the lower, undifferentiated, and early maturing sebocytes with decreased IR as sebocytes become more differentiated (Table S3, Figure 1b, S). This gradient was less obvious in the smaller sebaceous glands and IR was greatest during anagen I–V. The same gradient was also seen for Aldh1a1, Aldh1a2, Aldh1a3, cytoplasmic Rara, Rarb, and Rarg, but their IR level changed throughout the cycle (Table S3, Figure 1e, h, n, and q, S). Aldh1a1 first appeared during anagen I, peaked during anagen IIIb, and disappeared during anagen VI/catagen I. Aldh1a2 and Aldh1a3 were only seen weakly during anagen. Rars were seen in the sebaceous gland throughout the hair cycle, but peaked during early anagen. In contrast, Crbp and Crabp2 IR were greater in more differentiated maturing and mature sebocytes (Table S3, Figure 1k, S). This gradient was present throughout the hair cycle for Crabp2, but only during anagen I–V for Crbp. In addition, Crabp2, Rara, and Rarg were localized to the nucleus in the more-differentiated maturing and mature sebocytes in most stages with a peak in early anagen and a drop during anagen VI/catagen I (Table S3, Figure 1k and n, block arrow, Figure 3p and t). This nuclear localization was highly variable between hair follicles in the same stage, suggesting that it is a short event that is not always captured. Crabp2, Rara, and Rarg localized in both the cytoplasm and nucleus, whereas Rarb was only localized in the cytoplasm throughout the hair follicle, but was most obvious in the sebaceous gland.

RA biosynthesis enzymes, binding proteins, and receptors were present in the epidermis

Throughout the hair cycle, Dhrs9, Aldh1a2, Crabp2, Rara, Rarb, and Rarg were localized to the cytoplasm of the

epidermis (Table S1, Figure 1a, d, g, j, m, and p, yellow diamond). Weak Rara and Rarg IR was seen in the nuclei of the epidermis throughout the hair cycle, whereas Crabp2 was nuclear localized in some follicles during anagen IIIa and catagen V. Epidermis in mouse skin is thin, but when two layers could be distinguished Aldh1a2 was stronger in the basal/spinous layers, whereas Crabp2 was stronger in the granulosum/corneum layers (Table S1, Figure 1g and j, yellow diamond). The distinction between spinous and granulosum layers is hard to make in the mouse.

DISCUSSION

In this report, we show that all components necessary for RA biosynthesis and signaling are present throughout the hair follicle in a hair cycle-dependent manner (Figure 4). During telogen, Rars predominate in the cycling portion of the hair follicle (Figure 4a, Rars alone = purple). During anagen RA synthesis, enzymes and Crabp2 increased (Figure 4b and c, yellow = Dhrs9 and Rars, Aldh1a1-3 = orange, Crabp2 = blue), with the whole system necessary for RA synthesis and signaling present in several sites (Figure 4b and c, red). There was a temporary decrease in Aldh1a2 and Rars during anagen VI/catagen I, which was restored by catagen IV (Figure 4d and e, pink = Dhrs9 without Rars, orange = Aldh1a3). Crbp IR increased during anagen VI/catagen I and remained intense through catagen IV (Figure 4d and e, green). By catagen VII, Dhrs9 and Rars decreased in most of the cycling portion of the hair follicle (Figure 4f, decrease in yellow, pink, and purple). On the basis of the assumption that presence of enzymes, proteins, and receptors equates to RA synthesis and signaling (see Introduction and Results), this localization pattern suggests that RA plays many roles in the cycling hair follicle.

One role of endogenous RA may be to regulate the telogen to anagen transition. During telogen, the dermal papilla sits adjacent to the bulge, and signaling between the dermal papilla and stem cells triggers the initiation of anagen (Stenn and Paus, 2001; Alonso and Fuchs, 2003; Cotsarelis, 2006). As Rara can repress transcription in the absence of RA (Privalsky, 2004), its intense presence in the dermal papilla and cells outside the bulge along with reduced RA synthesis enzymes at this site during telogen suggest that RA target genes may be repressed during telogen, then activated during anagen when RA synthesis is predicted to occur. As RA can act by paracrine and/or autocrine mechanisms, it is unclear whether RA signaling is occurring in the dermal papilla, stem cells or those cells just outside the bulge (stem cell niche), or all three as Rars localized to all three cell types. Note that Crabp2 is not required for RA signaling, but makes it more efficient when RA levels are low (Dong *et al.*, 1999; Budhu and Noy, 2002; Sessler and Noy, 2005). Crabp2 may also be important when RA needs to be protected from degrading enzymes. Cytochrome p450, family 26, subfamily b, polypeptide 1, which degrades RA, localized to the dermis surrounding hair follicles in the embryo (Abu-Abed *et al.*, 2002). If the same localization pattern also occurs in the adult, it suggests that Crabp2 is acting to facilitate RA signaling in the niche cells during anagen, but not the rest

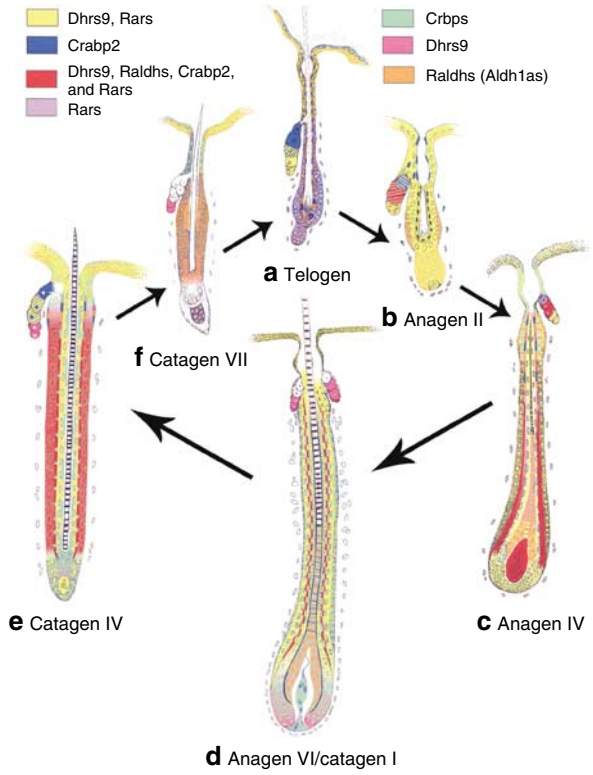

Figure 4. Localization patterns of enzymes, binding proteins, and receptors involved in RA synthesis and signaling throughout the hair cycle. (**a**) During telogen, Rars (purple alone, yellow with Dhrs9) predominate with occasional localization of Dhrs9 (yellow when with Rars, pink alone) and Raldhs (Aldh1as, orange), whereas Crabp2 (dark blue) localized within the bulge, sebocytes, inner layer of the outer root sheath, and stratum granulosum/cornium. Dashed and dotted lines on top of or along with another color indicate that both of those proteins were present in that layer. For example, the stripes of blue and orange in the inner layer of the outer root sheath indicate that both Crabp2 and Raldhs were seen in this layer and the orange highlights in the epidermis indicates that Raldhs, Dhrs9, and Rars were present in the stratum basalis/spinosum. (**b**) During anagen II, localization of Dhrs9 (yellow) and Raldhs (orange dots, dashes, and highlights) increased. Crabp2 now localized to cells outside the bulge, whereas Crbp (green dashes) and Raldhs (orange dashes) localized within the bulge along with Dhrs9 and Rars (yellow). In the sebaceous gland the complete system for RA synthesis and signaling (red + green) localized to the cytoplasm of differentiating cells, whereas Crabp2 and Rars were localized within their nuclei. (**c**) By anagen IV the complete system without Crbp (red) localized to the dermal papilla, lower companion layer, Henle's layer of the inner root sheath, as well as differentiating sebocytes. In addition to Crabp2 and Rars, Raldhs now also localized to cells outside the bulge. (**d**) During anagen VI/catagen I, localization of Rars dropped in the matrix, sebocytes, and connective tissue sheath (pink is Dhrs9 only) and localization of both Rars and Dhrs9 dropped in the dermal papilla and precortex/premedulla, whereas localization of Crbp and Crabp2 increased throughout the lower cycling hair follicle (green and dark blue lines, dashes, highlights, and red and green line). Crabp2 now localized to the outer edge of the bulge along with Dhrs9 and Rars. (**e**) During catagen IV, the complete system with Crbp localized to the outer root sheath and companion layer. The whole system without Rars was seen in the bulge, whereas Rars localized to cells outside the bulge. IR of Rars increased within the dermal papilla and sebaceous gland. Crabp2 and Rars also localized to differentiating sebocyte nuclei. IR of Raldhs dropped in the inner root sheath and hair fiber. (**f**) By catagen VII, IR of Dhrs9 and Rars dropped in many places and Crbp was only localized to the sebaceous gland ducts. This suggests that RA synthesis and signaling was most active during all of anagen and early catagen. The increase of Crbp during early catagen may also suggest an increase in retinyl ester formation (vitamin A storage), as Crbp is also involved in this process.

of the connective tissue sheath. In other tissues, RA regulates vitamin D receptor, bone morphogenic protein, and wingless related mouse mammary tumor virus (Wnt)-signaling pathways, which are involved in anagen initiation (Botchkarev *et al.*, 2001; Merrill *et al.*, 2001; Balmer and Blomhoff, 2002; Botchkarev and Kishimoto, 2003; Van Mater *et al.*, 2003; Zhuang *et al.*, 2003; Lo Celso *et al.*, 2004; Morris *et al.*, 2004; Paus and Foitzik, 2004; Shibamoto *et al.*, 2004; Shimizu and Morgan, 2004; Tumbar *et al.*, 2004; Lowry *et al.*, 2005).

During early anagen, RA may regulate the proliferation and migration of both epithelial and mesenchymal cells during bulb formation. The localization pattern of RA synthesis and signaling members throughout early anagen in proliferating/migrating keratinocytes was similar to that of various Wnt-signaling factors, as well as the Sonic hedgehog receptor *Patched homolog1* (Reddy *et al.*, 2001; Oro and Higgins, 2003; Reddy *et al.*, 2004). RA can have either antagonistic or synergistic effects on Wnt signaling (Mulholland *et al.*, 2005). Thus, RA may regulate Wnt signaling during epithelial cell migration and bulb formation as we suggested from Aldh1a2 localization (Everts *et al.*, 2004). In the connective tissue sheath Rara may either receive epithelial cell-produced RA or repress transcription in the absence of RA (Privalsky, 2004). The lack of Crabp2 and potential presence of cytochrome p450, family 26, subfamily b, polypeptide 1 (Abu-Abed *et al.*, 2002, see above) in these cells favors the hypothesis that Rara represes gene expression. Future studies are needed to examine the localization patterns of RA-degradative enzymes and Crabp type 1 throughout the hair cycle, as well as determine what genes are repressed by unliganded Rara.

Later in anagen, endogenous RA may regulate the differentiation of all layers of the hair follicle. Matrix cells are believed to differentiate into the hair fiber as well as the inner root sheath and companion layer, which enclose and support the hair fiber (Langbein and Schweizer, 2005, Legue and Nicolas, 2005). This process is regulated by signaling between the matrix cells and the mesenchymal dermal papilla, as well as signaling within these differentiating cells (Botchkarev and Kishimoto, 2003; Paus and Foitzik, 2004; Cotsarelis, 2006). The presence of RA synthesis and signaling molecules in more differentiated layers of the hair follicle, as well as the bulb, is consistent with a role for RA in differentiation of three of these layers. RA may be acting in an autocrine manner in the dermal papilla during anagen IIIc and IV as the whole system is present (Figure 4c, red), or during other stages retinal may remain in these matrix cells as they differentiate and move upward to be converted into RA by Aldh1a3 later during differentiation. Alternatively, retinal may be synthesized in the epithelial matrix cells, move to the mesenchymal dermal papilla to form RA, which then acts back in the epithelial matrix cell. We found a similar type of RA biosynthesis and signaling sequence in the rat uterus (Li *et al.*, 2004). In addition, RA appears to act only in a paracrine manner during eye development (Molotkov *et al.*, 2006). Differences in Aldh1a2 and Aldh1a3 localization between this study and our previous report (Everts *et al.*, 2004) are due, in part, to the fact that we examined more hair follicles in this report. In addition, we used a different, more sensitive, antibody against Aldh1a3 in this study (see Materials and Methods). Formation of these layers involves several factors regulated by RA in other tissues, including Wnt and bone morphogenic protein pathways, and keratins 6a and keratin 75 (also known as K6hf) (Rosenthal *et al.*, 1992; Millar *et al.*, 1999; Balmer and Blomhoff, 2002; DasGupta *et al.*, 2002; Li *et al.*, 2003; Paus and Foitzik, 2004; Smyth *et al.*, 2004; Baron *et al.*, 2005). In addition, Aldh1a3 expression in the hair fiber medulla was reduced in dominant-negative fibroblast growth factor 7 and fibroblast growth factor 10 receptor *Fgfr2* mice (Schlake, 2005), further supporting a role for RA in hair medulla differentiation.

In contrast to the differentiation of the companion layer, inner root sheath, and hair fiber, recent data suggest that the outer root sheath develops during each cycle by differentiation directly from the bulge stem cells and scattered apoptosis (Langbein and Schweizer, 2005; Legue and Nicolas, 2005). The downward gradient of RA synthesis and Rarb from the bulge region is consistent with a role for endogenous RA in outer root sheath differentiation. Crabp2-facilitated RA signaling may regulate this scattered apoptosis, as Crabp2 has been implicated in apoptosis regulation in mammary cells (Donato and Noy, 2005).

Another role of endogenous RA may be to regulate catagen, as there were several changes in the RA synthesis and signaling localization pattern during the anagen to catagen transition. These include a switch in Crabp2 localization from the cells outside the bulge (stem cell niche) to cells within the bulge (stem cells); a decrease in Rara in the dermal papilla and connective tissue sheath, except those outside the bulge (stem cell niche); and an increase in Crbp and Crabp2 within the lower, regressing, follicle. In addition, exogenous RA given to anagen hair follicles in culture induced premature catagen via transforming growth factor β 2 (Foitzik *et al.*, 2005). The switch in Crabp2 localization may reduce RA signaling in the stem cell niche, whereas increasing RA signaling within the stem cells. Transforming growth factor β 2 may be among the stem cell genes altered by this switch (Morris *et al.*, 2004; Tumbar *et al.*, 2004). When exogenous RA is given during anagen, it may disrupt the balance of RA and binding protein in the bulge stem cells allowing gene expression to occur in the absence of control by Crabp2, leading to premature catagen. During anagen, Rara may act to repress transcription within the connective tissue sheath (see above). The decrease of Rara in the connective tissue sheath during anagen VI/catagen I would remove this inhibition and trigger catagen. In addition to the reported transforming growth factor β2 involvement, intracellular adhesion molecule 1 may also be involved. Intracellular adhesion molecule 1 localization was opposite that of Rara; it activates catagen, it contains a functional retinoic acid response element, and RA regulates intracellular adhesion molecule 1 in normal human epidermal keratinocytes in culture (Aoudjit *et al.*, 1995; Janssens *et al.*, 1999; Muller-Rover *et al.*, 2000). When exogenous RA is given during anagen, it would disrupt the balance of liganded to unliganded Rara and activate transcription of RA target genes such as transforming growth factor β 2 and intracellular

adhesion molecule 1 to induce premature catagen. Crbp facilitates the conversion of retinol into both RA and retinyl esters (Napoli, 1999; Ong, 1994). Both RA and retinyl ester synthesis may be occurring during catagen, as RA synthesis enzymes are still present, but have not increased. RA and Crabp2 may be regulating apoptosis (Donato and Noy, 2005), whereas the excess retinol is diverted to storage. Later in catagen, RA synthesis and signaling decreased, which may inhibit cell growth and limit apoptosis.

As retinoids dose dependently regulate sebocyte proliferation and lipogenesis, and are one of the major forms of treatment for acne vulgaris, it was not surprising to see the RA synthesis and signaling pathway localized there (Zouboulis, 2001, 2004). What is novel is the localization pattern, which suggests that RA synthesis occurs in the less differentiated sebocytes, but activation of gene expression in the nucleus occurs in more differentiated sebocytes where little to no RA synthesis is occurring. These observations have two implications. The first implication is that RA regulates sebocyte proliferation from the more differentiated cells. RA may regulate proliferation via Indian hedgehog, as Indian hedgehog expression was changed by altered levels of RA in other tissues and Indian hedgehog is expressed in differentiated sebocytes but acts on undifferentiated sebocytes (Yoshida *et al.*, 2001; Wu *et al.*, 2002; Niemann *et al.*, 2003; Bohnsack *et al.*, 2004).

The second implication of this localization pattern is that translocation of Crabp2 and Rars to the nucleus is a regulated process. In cultured cells, Crabp2 translocates to the nucleus within minutes of RA addition and binding of RA to Crabp2 reveals a nuclear localization signal (Dong *et al.*, 1999; Budhu and Noy, 2002; Sessler and Noy, 2005). Crabp2 can also bind Rara, channeling RA from Crabp2 to Rara, and increase the efficiency of RA-induced transcription (Delva *et al.*, 1999; Budhu and Noy, 2002; Donato and Noy, 2005). Our study confirms the nuclear localization of Crabp2 in a whole animal, but more importantly suggests that in the whole animal Crabp2 requires more than just RA for nuclear localization. What keeps Crabp2 and Rars from the nucleus in less differentiated sebocytes as RA is presumably present? What then triggers nuclear translocation in differentiated sebocytes? In the testis, nuclear localization of Rara and activation of a retinoic acid response element reporter gene were inhibited by follicle-stimulating hormone, via protein kinase A, and peroxisome proliferators, and stimulated by protein kinase C (Dufour and Kim, 1999; Braun *et al.*, 2000, 2002; Dufour *et al.*, 2003). In addition, using green fluorescent protein labeling, several other nuclear steroid receptors were found to shuttle between the nucleus and cytoplasm (Kawata, 2001). RA and Rars may also act in the cytoplasm (Everts and Berdanier, 2002; Canon *et al.*, 2004; Rochette-Egly, 2005). Additional studies are required to determine what regulates the nuclear localization of Crabp2 and Rars in the hair follicle and sebaceous gland and what role, if any, RA and Rars have within the cytoplasm.

Endogenous RA may also regulate epidermal differentiation and epidermal permeability barrier function. RA regulates several genes in keratinocytes including the differentiation markers transglutaminase, involucrin, filaggrin, keratin 6a, and keratin 13 (Baron *et al.*, 2005; Rosenthal *et al.*, 1992). But, in the suprabasal epidermal layer targeted mutations revealed an essential role for unliganded Rarg/Rxra and liganded peroxisome proliferators activator-receptor δ/Rxra in lamellar granule regulation and epidermal permeability barrier function (Calleja *et al.*, 2006). Yet, we saw Rarb in the cytoplasm throughout the epidermis. As Rarb is upregulated by RA (Haq *et al.*, 1991), it suggests that active RA signaling is occurring throughout the epidermis. Studies in cultured primary keratinocytes also suggest that active RA signaling may occur in the suprabasal layer with induction of differentiation (Vettermann *et al.*, 1997; Chatellard-Gruaz *et al.*, 1998). How could Rarg remain unliganded when RA synthesis is occurring within this layer? We propose that Crabp2 binds some of the synthesized RA and directs it to Rara, whereas the rest of the RA is degraded. Crabp2 directs RA to Rara and transcriptional activation (Noy, 2000), but Crabp2 binding to Rarb and Rarg have not yet been tested and may not occur. During lung development, Rara is required to maintain Rarb, whereas Rarb regulates fibroblast growth factor 10 expression and lung morphogenesis (Desai *et al.*, 2006), suggesting that different Rars regulate different sets of genes. In addition, the cytochrome p450, family 2, subfamily S, polypeptide 1 localized throughout the epidermis except the cornified layer in human skin, is upregulated by RA and degrades RA (Smith *et al.*, 2003; Saarikoski *et al.*, 2005), suggesting that any excess RA not protein-bound in the epidermis could be degraded. Thus, endogenous RA may activate Rara-specific genes, like *Rarb*, whereas Rarg remains unliganded. The cytoplasmic Rarb may also limit the delivery of RA to Rarg. Exogenous RA would alter this balance of Crabp2-directed transcription and activate Rarg target genes resulting in hyperproliferation and skin fragility.

In summary, this localization pattern suggests that RA regulates epithelial–mesenchymal interactions in the stem cell niche and bulb, anagen induction, and differentiation of all layers of the pilosebaceous unit during anagen. RA synthesis may occur in less differentiated cells, but signaling occurs only with differentiation. RA may induce catagen by reduced unliganded Rara in the connective tissue sheath and localization of RA signaling within the bulge, as well as activate apoptosis throughout the regressing follicle. The localization of endogenous RA synthesis and signaling also revealed sites where exogenous RA could disrupt the balance of liganded *versus* unliganded Rara and Crabp2 leading to the reported toxic effects including hyperproliferation of the epidermis, skin fragility, and hair loss. This highlights the need for a better understanding of endogenous RA function and the regulation of endogenous RA synthesis, which could lead to better treatments without unwanted side effects. Additional functional studies should examine the function of endogenous RA and the consequences of altered RA synthesis and signaling in the pilosebaceous unit.

MATERIALS AND METHODS

Tissue acquisition

Twenty-four female C57BL/6J mice were wax stripped to induce anagen, as described previously (Muller-Rover *et al.*, 2001). Mice

were anesthetized with 2% tribromoethanol solution (IP, 0.2 ml/10 g; Sigma-Aldrich, Milwaukee, WI) and all dorsal skin follicles in telogen, as indicated by pink color, were painted with warm wax (Surgi-wax; Ardell International, Los Angeles, CA) that was peeled off after hardening, thus depilating all dorsal skin hair fibers. Skin samples were harvested from two C57BL/6J wax stripped mice at each of the following time points: 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 32 days after depilation. Skin for both routine histology and immunohistochemistry (IHC) was fixed overnight in Feketes acid–alcohol–formalin solution (61% ethanol, 3.2% formaldehyde, 0.75 N acetic acid), transferred to 70% ethanol, processed routinely, embedded in paraffin, sectioned at 5–6 μm, placed on microscope slides (Superfrost/Plus Fisherbrand, Pittsburgh, PA) and stained with hematoxylin and eosin for routine histopathologic analysis. All mouse studies were approved by The Jackson Laboratory Institutional Animal Care and Use Committee and were performed in compliance with stipulations of that body.

Antibodies

All rabbit studies were approved by The Vanderbilt University Medical Center Institutional Animal Care and Use Committee and were performed in compliance with stipulations of that body. Antibodies against Crbp, eRoldh/Dhrs9, Aldh1a2, and Aldh1a3 were all produced within our laboratory and their specificity was established previously (Zheng and Ong, 1998; Rexer and Ong, 2002; Everts *et al.*, 2004). The antibody used against Aldh1a3 in this study was from a different rabbit raised at the same time as the antibody described previously (Everts *et al.*, 2004). This antibody proved to be more sensitive for IHC, whereas the specificity based on dot-blot analysis against bacterially expressed proteins was the same. Antibodies raised against different epitopes of eRoldh/Dhrs9 and Crabp2 were examined and produced similar results. The first of these two antibodies has been described previously (Wardlaw *et al.*, 1997; Rexer and Ong, 2002). The second antibody raised against a different epitope of Crabp2 was produced as described previously (Everts *et al.*, 2004) using the peptide CEQRLLKGEGPKTS from the rat sequence. Two additional antibodies raised against different epitopes of eRoldh/Dhrs9 were produced as described previously (Rexer and Ong, 2002) using the peptide CGFLWTRKGKLKIED from the human sequence and DPIKTTEKKLA from the rat sequence. Specificity of the antibodies against Crbp, eRoldh/Dhrs9, Aldh1a2, Aldh1a3, and Crabp2 were also confirmed by obtaining similar localization patterns between IHC, Western blot analysis, and reverse transcription-PCR in sites that have been previously shown to be sensitive to dietary vitamin A (Everts *et al.*, 2005). In that study, we also found eRoldh/Dhrs9 and Crabp2 to be negative in rat liver and kidney by all three methods. The Aldh1a1 antibody was also produced as described previously (Everts *et al.*, 2004) using the peptide CGGGRWGNKGFFVQP from the rat sequence. Protein levels of Aldh1a1 (Raldh1) by both Western blots analysis and IHC are greater in the uterus with this antibody during diestrus than estrus (unpublished observation), which is the same localization pattern as we reported for the mRNA (Li *et al.*, 2004). Specificity for antibodies against Crbp, eRoldh/Dhrs9, Aldh1a1-3, and Crabp2 was also confirmed by running IHC with equivalent amounts of IgG (determined by absorption at 280 nm) from the IgG flow through from the respective affinity columns. Except for the antibody against eRoldh/Dhrs9 (whose specificity was confirmed by several other methods, see above) there was no IR seen in sites where the affinity-purified antibody was seen, and some IgG fractions were completely negative (data not shown). Antibodies against Rara, b, and g were purchased from Santa Cruz Biotech (Santa Cruz, CA). To confirm their specificity, these antibodies were preabsorbed for 2 hours at room temperature with five times their respective blocking peptide (Santa Cruz Biotech). The expression seen for both Rara and Rarb was completely lost in the control that was preabsorbed with blocking peptide. But there was still some background signal seen after preabsorption with the Rarg antibody. This background signal was subtracted from the signal seen without the peptide to determine the expression level of Rarg, although some sites were not scorable with the Rarg antibody because of this background. Controls that used just blocking solution (no primary antibody) were also run. Occasionally background signal was seen in sebaceous glands in the no primary control sections.

IHC

Sections were pretreated with 3% hydrogen peroxide, blocked with 3% BSA plus 1.28% normal goat serum, incubated with affinity-purified rabbit polyclonal antibodies against Crbp, eRoldh/Dhrs9, Aldh1a1, 2, or 3, Crabp2, Rara, b, or g (described above) overnight at 4°C. Tissue was then incubated with a biotin-conjugated secondary antibody, an anti-biotin IgG-conjugated with horseradish peroxidase, and then stained with AEC+ (Dako, Carpinteria, CA; Bucco *et al.*, 1997). Hydrogen peroxide and BSA were obtained from Sigma . Normal goat serum, secondary, and tertiary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Hair follicles were classified by stage (Muller-Rover *et al.*, 2001) and IR scored on a scale of 0–4, blinded by the knowledge of days after depilation. For most stages, three to nine hair follicles were scored per stage. During anagen VI/catagen I and late catagen through early anagen, 11–24 hair follicles were scored per stage, but only one or two follicles in catagen II and III were seen and scored. Mean and SD was performed to highlight variability, but no statistics were run as this study was intended to point out qualitative localization patterns.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Immunolocalization of RA biosynthesis and signaling primarily with Aldh1a2.

Table S2. Immunolocalization of RA biosynthesis and signaling with primarily Aldh1a3.

Table S3. Immunolocalization of RA biosynthesis and signaling using various retinal dehydrogenases.

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