Expression of Morphogens During Human Follicle Development In Vivo and a Model for Studying Follicle Morphogenesis In Vitro

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Hair follicles form in developing skin as products of precisely timed and located interactions between the fetal epidermis and specialized populations of dermal cells. The follicle, per se, is the epithelial component only, but the structure we are interested in is the aggregate of the follicle, its associated mesenchymal cells, basement membrane and mesenchymally derived fibrous sheath, and dermal papilla. Discussion of the developing "follicle" assumes this composite structure and these various elements are partners in the epithelial-mesenchymal interactions that contribute to its formation. Epithelial-mesenchymal interactions also occur during development to construct organs (e.g., the kidney, gut, and lung), and glands (e.g., thyroid, mammary and salivary glands) and to mold other epidermally derived appendages such as the eccrine sweat glands, nails, feathers, scales, and vibrissae. What has been learned about the tissue interactions during development of these organs and structures provides insight into follicle morphogenesis in skin (reviewed in [1]).

Follicle morphogenesis begins with an inductive event that involves the exchange of signals between epithelial and mesenchymal cells, then proceeds through the stages of initiation, elongation, and differentiation. These stages have been well described morphologically [2] and some of the interactions that occur have been identified (e.g., [3-5]). The challenge is to extend our understanding beyond the descriptive properties of the cells and tissues involved to determine how the physical and molecular signals trigger induction, then promote and regulate proliferation and changes in cell shape, adhesion, and migration necessary to continue morphogenesis, stimulate differentiation, and, finally, provide an environment that permits the differentiated structure to be maintained. Unanswered questions about this complex process include the following. What stages of follicle development involve epithelial-mesenchymal interactions and in what regions of the follicle are they likely to occur? When are instructive messages that provide specific directions exchanged? When are permissive messages that support continued morphogenesis in the direction it is headed (and in a manner already determined) exchanged? How are the messages transmitted? Are messages more precise and targeted as development progresses? And, if so, does this explain the difficulty of the follicle to continue development in our in vitro model? Are there parallels between the events and factors that underlie follicle embryogenesis and those that regulate phases of the hair cycle once the follicle is fully formed?

The respective roles of the epidermis and dermis in follicle morphogenesis have been defined from classic studies in which separated epidermis and dermis, mismatched in age, body site, species, genetic type, etc., are recombined and placed in culture or grafted to an animal [6]. The epidermal appendages that form in these tissue recombinants have been analyzed structurally, immunohistochemically, and biochemically. Such studies have demonstrated that the dermis provides a substrate and the directions to change an undifferentiated ectoderm into an epidermis, then regulates its proliferation, stratification, and keratinization, thereby determining the resultant epidermal architecture. In response to signals from the epidermis, the dermis initiates epidermal appendage morphogenesis and determines the pattern and properties (size and shape) of the appendage according to its own regional specificity. The epidermis determines the type of the appendage, directs its cephalic causal polarity, and determines the species specificity of the keratin composition and the morphology of corneocytes [6].

The morphology of the follicles that form in the tissue recombinants and in normal developing skin suggests that the epidermis and dermis communicate during this process through cell-cell contact [5,7,8], cell-matrix interactions [9], and an association of the interacting tissues with nerves [10,11]. Both the physical contact and the "nontouching" relationships between epithelial and mesenchymal cells are certain to involve molecules, called "morphogens," that play a regulatory role in development: cell adhesion molecules (CAMS or cadherins) and other cell-surface associated molecules (e.g., glycolipids and proteoglycans), growth factors (GFs), extracellular matrix molecules, hormones, cytokines, enzymes, and certain pharmacologically relevant molecules, e.g., retinoids, and their receptors. By determining which of these molecules are present in the environment of the developing skin and the microenvironment of the developing follicle, and which are developmentally regulated (expressed with tissue and stage specificity), it is possible to identify candidate molecules that might be involved in follicle morphogenesis. Their importance in this process then can be tested using an in vitro model that supports follicle morphogenesis. This approach has been effective in identifying factors that are critical for certain events to occur (e.g., condensation of dermal mesenchyme cells) during follicle morphogenesis in developing rodent skin [4,5] and feather morphogenesis in the embryonic chick [12]. To our knowledge these studies have not been accomplished in human fetal skin, perhaps because of the lack of a model system that will support follicle initiation and development.
The goals of this overview of follicle morphogenesis are 1) to review the stages of follicle development in vivo in human skin, incorporating new data with published data from our laboratory [13-15] and from others on the developmental expression of selected "morphogens" that may participate in the epithelial-mesenchymal interactions, and on structural proteins of the follicle and its associated mesenchyme that may reflect the interactions, and thus the status of differentiation, and, 2) to describe studies of human fetal skin explants in a suspension organ-culture system that supports human fetal skin development and some of the early events of follicle morphogenesis.

THE PREFOLLICULAR TISSUE ENVIRONMENT AND INDUCTION

Because we do not know the precise timing of follicle induction and whether there is a lag between induction and follicle initiation, we need to understand the condition of the skin within the time period that precedes follicle initiation. This period coincides with the embryonic-fetal transition at 2 months' estimated gestational age (EGA). At this stage, the two-layered epidermis (periderm and basal cells) of the embryo (less than 60 d EGA) stratifies and the dermal-epidermal junction (DEJ) changes from a simple basal lamina to a basement membrane zone that includes attachment structures and antigens associated with adhesion. The cellular matrix of the thin embryonic dermis begins to accumulate fibrous matrix. Each region of the skin expresses many of the structural proteins and antigens that are characteristic of postnatal skin, even though the structure of the skin at this early fetal stage (60-70 d EGA) bears little resemblance to adult skin [16].

The basal and intermediate layers of the stratified epidermis express the keratins typical for the basal and suprabasal layers in adult skin as well as periderm-characteristic keratins [17,18]. Epidermal growth factor receptors (EGFR) are expressed on both basal and intermediate layer keratinocytes [14], the A-chain of platelet-derived growth factor (PDGF) is expressed by basal cells, and the B-chain of PDGF is present in the cytoplasm of cells in the intermediate layer. The embryonic and fetal keratinocytes express E-cadherin [19], but only the embryonic epidermal keratinocytes express neural cell adhesion molecule, N-CAM. Immunostaining of the basement membrane zone reveals the presence of heparan sulfate proteoglycan (HSGP), chondroitin 4- and 6-proteoglycans (C4-SPG, C6-SPG), and type IV collagen.

Several fibrous and filamentous matrix molecules (e.g., types I, III, V, VI, and VII collagen [20]) and fibrillin are also concentrated in the reticular lamina, a thin zone of fibrous and diffuse matrix immediately beneath the basal lamina. This layer of matrix causes the basement membrane to appear "thickened" in immunostained preparations examined by light microscopy. A broader, second subepidermal zone of dermis (that includes the reticular lamina) is the "compact mesenchyme" (Fig 1), a zone that is rich in cells that express growth factor receptors, cell adhesion molecules, and nerves. We have observed enhanced immunostaining for p75 (a neurotrophin receptor, see below), neural cell adhesion molecule (NCAM), and the PDGFR-alpha (PDGFRα) and PDGFR-beta (PDGFRβ) subunits in this mesenchyme of human embryonic and early fetal skin. The compact mesenchyme in the developing skin of other species is rich in fibroactin [22,23], transforming growth factor-β (TGF-β) [24,25], acidic and basic fibroblast growth factor (aFGF and bFGF) [26,27], bFGF receptor (bFGFR) [28], bone morphogenetic protein-2a or -4 mRNA (BMP-2a, BMP-4) [29,30], and hyaluronic acid (HA). Some of the molecules distributed generally throughout the compact mesenchyme prior to follicle initiation become restricted in distribution to the follicle-associated mesenchyme thereafter (e.g., p75, N-CAM). Those that remain distributed generally in the compact mesenchyme may reflect the proximity of this tissue to the epidermis.

STAGES OF FOLLICLE MORPHOGENESIS

Initiation: Formation of the Follicular Germ and Condensation of Follicle-Related Dermal Mesenchyme (Fig 2 and

![Figure 1. Skin from the arm of a 52-d EGA human embryo showing a concentration of fibroblastic cells immediately beneath the epidermis (the compact mesenchyme) (a) and the differential reactivity of these cells with an antibody to PDGFRα. Magnification X285.](image)

![Table 1. Follicle morphogenesis is initiated in vivo ~80 d EGA, recognized morphologically by regularly spaced clusters of basal keratinocytes that form an epidermal placode or follicle germ [2]. The epithelial germs are matched across the basal lamina by mesenchymal cells that are thought to aggregate in response to epidermal signals and to involve the synthesis and cell surface presentation of new proteins [5]. The cells of the follicle, condensed mesenchyme, and the basement membrane between them, have different properties compared with the keratinocytes, basement membrane, and mesenchymal cells of the interfollicular skin.](table)

![Figure 2. Diagram of the follicle germ illustrating the condensing mesenchyme proximal to the epidermally-derived follicle cells.](image)
Table I. Comparison of the Expression of Selected Growth Factors and Receptors, Cell Adhesion Molecules, Structural Proteins, and Matrix Molecules by Cells of the Follicular and Interfollicular Epithelium, the Basement Membrane Between the Two Regions, and the Related Mesenchymal Tissue of the Follicle Germs

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*1, intermediate layer cells; B, basal cells; C, core; O, outer layer cells.

the basement membrane beneath the germ is continuous with that of the interfollicular epidermis and shows no change in the expression of types IV and VII collagen, laminin (LN), or HSPG, or in the morphology or density of attachment structures. There are, however, follicle-specific additions and deletions of molecules to the basement membrane: C6-SPG and tenasin (TN) are strongly expressed in the basement membrane beneath both the pregerm and the germ but absent beneath the interfollicular epidermis [36,37]. Many of the antibodies (e.g., anti-TN) that appear to detect the basement membrane beneath the follicle also react with the associated matrix. Thus, the precise location cannot be assured. The composition of the basement membrane is important in that some of the molecules can bind matrix proteins and growth factors (e.g., HSPG binds members of the FGF family, LN, FN, and type IV collagen) are a reservoir for certain molecules (e.g., bFGF) or a conduit of communication between epithelial and mesenchymal cells.

Figure 3. Light micrographs of immunostained sections of fetal skin between ages 80 and 90 d EGA showing differential reactivity of germ-associated matrix and basal lamina when reacted with antibodies against types I (a), III (b), V (c), and VI (d) collagen. Types I, III, and V are reduced or absent in the matrix surrounding the germ and basal lamina compared with the immunostaining with these antibodies in the dermal matrix and interfollicular basal lamina. There is less of an apparent difference in immunoreactivity with type VI collagen throughout the dermis than with the other collagen antibodies. Magnification X 150.
Tenascin is, in fact, considered to be a marker for epithelial-mesenchymal interactions. The epithelium is suggested to produce a soluble factor that stimulates TN synthesis. In the developing gut, cells of the differentiated gut epithelium stimulate subjacent mucosal cells to synthesize TN [48] and the dental epithelium induces dental mesenchyme to synthesize TN in the developing tooth [49]. Tenascin in the follicle-associated matrix may act on the follicle epithelium or on the mesenchymal cells themselves. It has been suggested to promote mesenchymal cell aggregation and epithelial cell growth, but not attachment [50]. Tenascin also may interact with follicle keratinocytes through an association with proteoglycans in the basement membrane; in the developing rodent follicle it might communicate with epidermis through the syndecan matrix receptor [45]. In adult tissues, TN is present in the papillary and adnexal dermis but apparently reduced in expression, except in situations such as tumors and wound repair where there is active proliferation and growth, sites where TN could influence tissue morphogenesis [50,51]. The directions tenascin provides and the functions it regulates in the developing follicle are not known. N-CAM, like TN, is concentrated around the follicles and diminished in the general compact mesenchyme.

The dermal condensations are also focal sites of p75 expression [22], a molecule that binds all five members of the neurotrophin family of proteins that are structurally related to NGF (Fig 4). p75 is thought to be a component of high affinity, biologically active neurotrophin receptors when expressed coordinately with a tyrosine kinase product of a trkA or trkB proto-oncogene [52,53]. The specificity of the receptors for one or more of the neurotrophins depends upon the specific tyrosine kinase. We don't know if the trk genes are expressed in human follicle-associated mesenchyme, but Bothwell (personal communication) has demonstrated that mRNAs for neurotrophin 3 (NT-3) and perhaps brain-derived neurotrophic factor (BDNF) are synthesized by mesenchymal cells of the developing rodent follicle.

Neurotrophic factors present in the environment of the developing follicle may have multiple functions. Following the model of other systems, they could sustain sensory innervation to the follicle during development [11]. In this regard, immunostaining with p75 also reveals the close juxtaposition of nerves to the developing follicles (Fig 4) and the epidermis is both a site and a source of NGF [11]. Others [54] have also shown that mesenchymal cells in a variety of developing organs and pathologic tissues express p75, suggesting that they may be modulated by neurotrophic factors directly rather than through their innervation. The NGF receptor-ligand complex is mitogenic in some systems, but this role in follicle morphogenesis would seem to contradict the work of Wessells and Roessner [55] showing that the condensed, follicle-associated mesenchymal cells are nonproliferative. Using immunoelectron microscopy [22] we have determined that both N-CAM and p75 are expressed on the plasma membranes of the follicle-associated mesenchymal cells, thus both molecules may promote cell adhesion and continued accumulation of cells in this aggregation [56,57].

A role for the p75 receptor in the follicle-associated mesenchyme is suggested from preliminary experiments in which explants of 64-d, 67-d, and 78-d fetal skin were grown in suspension organ culture in the presence of 50 ng/ml NGF and sampled after 21, 27, and 35 d in culture. By day 27 follicles were initiated in the cultures of 67-d and 78-d skin samples (Figs 5a,b). The tissue appeared to develop normally in the presence of exogenous NGF except for dispersion of p75-positive, follicle-associated mesenchymal cells (Figs 5c,d). They remained, however, within a domain of the follicle, suggesting that there is structural order to this accumulation of cells beyond their own adhesiveness.

Expression of both PDGFR subunits on the follicle-associated mesenchymal cells, and, to a lesser extent on the subepidermal fibroblastic cells, and both A and B chains of PDGF (Fig 6) within the epidermis and follicle epithelium of developing human skin suggests that this growth factor and its receptors may be involved in epithelial-mesenchymal interactions in the developing skin. The A-isoform of PDGF [58] and its corresponding α-receptor [59,60] are important in the development of the epidermis (A-chain) and dermis (α-receptor) of mouse skin. Animals with the Patch (Pch) mutation lack the α-receptor. This is frequently a lethal mutation; surviving animals lack a dermis [59,60]. PDGF is a mitogen for mesenchymal cells and a chemoattractant for smooth muscle cells, fibroblasts, and other connective tissue cells; it stimulates collagen and collagenase synthesis, mobilizes Ca++, and stimulates tyrosine-specific phosphorylation and phosphatidylinositol turnover [61,62]. Any of these functions could promote follicle morphogenesis, although not necessarily specifically. The synthesis of PDGF has also been correlated with the downregulation of EGF receptors in some systems [63] and in others it interferes with 125I-EGF binding with-

Figure 4. A section of 100-d EGA fetal skin that has been double labeled with DAPI fluorescent stain for nuclei (a) and the p75 antibody (b) recognizing the low-affinity NGFR receptor. Note the strong positive immunoreactivity of the mesenchyme associated with the germ and the nerves in surrounding dermis. Magnification X 270.

Figure 5. Light micrographs of 67-d EGA fetal skin grown for 27 d in suspension organ culture in control medium (a,b) or in 50 ng/ml NGF (c,d). The skin lacked germs at the time of explant. The follicles were initiated in culture (a,b). Follicles were also initiated in the NGF-treated cultures but neither the mesenchymal cells nor the follicular epithelial cells retained their normal association (b,d). (a) magnification X 90; (b,d) magnification X 180.
out a decrease in numbers of EGF receptors [64]. The latter observation is in accord with reduced 125I-EGF binding to follicular epithelial cells of developing rodent follicles [31] and with our studies of human follicle morphogenesis [14]. The decreased affinity for EGF may be an advantage to continued follicle development in the human if, as in the rodent, EGF delays the onset of follicle formation [65]. The strong and specific immunostaining patterns of the receptor and ligand in fetal skin contrast with expression in adult skin, where the receptor is expressed at a low constitutive level unless induced by inflammation [66, 67].

**Follicle Elongation: The Follicle Peg Stage** (Fig 7 and Table II)

The follicle elongates as a cord of cells that grows into the dermis at an angle relative to the epidermis, still in contact with mesenchymal cells along all surfaces. The outer cells of the hair peg are continuous with the basal epidermal layer. Cells within the core of the follicle are elongated lengthwise, perpendicular to the outer layer cells. At the end of the peg stage the tip of the follicle has flattened, defining (morphologically) the presumptive matrix of the follicle. How the matrix is established from cells that appear homogeneous, and how matrix cells divide in precise patterns to give rise to concentric layers of cells of the inner root sheath and hair fiber is unknown. The matrix cells are assumed to be under the control of mesenchymal cells in the dermal papilla but there is no sufficient explanation for the means by which these cells give such precise directions. A narrow cylinder of cells in the upper region of the peg is different morphologically and immunohistochemically from the more distal region of the peg. PDGF-A chain antibody, for example, reacts more strongly with upper region cells than with those of the lower region.

The outer layer follicle cells express the K5, K14 basal cell keratins, K17, K6, and K19 [17, 18]. K19 is reduced in the peg [13] and will become localized even more specifically as the follicle begins to differentiate. EGFRs remain absent or masked and ICAM-1 is no longer expressed by the follicle epithelial cells. PDGF-A and -B chains continue to be expressed in the basal and suprabasal layers of the interfollicular epidermis and the outer (A chain) and inner (B chain) cells of the follicle. The antibody to E-cadherin remains strongly expressed in the intermediate cells of the epidermis and in all cells of the follicle except the presumptive matrix, which shows a distinct absence of label. Ultrastructural studies reveal differences between the keratinocytes of the hair peg proximal to the epidermis and the core cells of the germ. Magnification X 300.
compared with those of the developing matrix. Bundles of keratin filaments are absent from the matrix and K14 mRNA expression is lacking [68].

The basement membrane of the hair peg remains strongly immunopositive for HSPG and C6-SPG but lacks C4-SPG. Type IV collagen, LN, and type VII collagen are continuous in the basement membrane zone of both the follicle and the interfollicular epidermis. Studies of embryonic rat skin have revealed that bulbous pempoid antigen (BPA), a molecule associated with hemidesmosomes [36] and therefore involved in the attachment of epidermal cells to the basement membrane, is reduced or absent at the distal end of the elongated follicle, suggesting that the hair peg lacks hemidesmosomes. Electronmicroscopy of the human follicle reveals that hemidesmosomes are decreased in distribution and reduced in structure but not absent. Diminished BPA expression, reduced hemidesmosomes, and decreased fibrous matrix in the reticular lamina (see below) may permit greater flexibility between the epidermis and DEJ to accommodate the downgrowth of the follicle yet maintain sufficient integrity of the DEJ to assure that the epithelial cells are properly assembled and oriented.

Follicle Peg-Associated Mesenchymal Cells: All follicle-related mesenchymal cells continue to be TN-rich, and express N-CAM and PDGF-B, but they are beginning to sort out into collections of cells that acquire different phenotypes according to the region of the follicle. Some of the cells remain associated with the lateral surface of the follicle and form the follicular sheath. Aggregated mesenchymal cells associate with the flattened tip of the follicle as the presumptive dermal papilla. Only minor amounts of fibrous matrix contribute to the sheath. Types I and V collagen are not recognized in the sheath but immunostaining for types III and VI collagen is weakly (type III) or more strongly (type VI) immunopositive. Type I collagen is also absent from the matrix surrounding cells of the condensed mesenchyme but types III and VI collagen are expressed. Type V collagen is evident in the basement membrane zone beneath the presumptive follicle matrix and in spaces between the follicle-related mesenchymal cells. Fibrillin is present in the follicle-associated mesenchyme and the dermal matrix. The orientation of the fibrillin filaments follows the direction of the cells and thus defines the boundary between the follicle sheath and the dermis.

N-CAM is concentrated in the matrix surrounding the cells of the sheath below the neck of the follicle and reduced in the mesenchyme beneath the interfollicular epithelium. This may indicate that N-CAM is associated only with regions of the epithelium that are still actively supporting morphogenesis. In contrast, TN is more concentrated in the neck of the follicle and is less apparent, although present, in the condensed mesenchyme at the follicle tip. The “neck” will become the infundibulum of the differentiated follicle. The mesenchymal cells and the epithelial cells at the flattened tip of the follicle are an epithelial-mesenchymal unit that interacts to promote and sustain follicle differentiation. Hardy [69] has described this interaction as the “second dermal message” in which the mesenchyme tells the follicle epithelium “to make a hair.” The chemical nature of this interaction is unknown although in rodent tissue it appears to involve direct physical contact. Gaps are formed in the basement membrane and processes of epithelial and mesenchymal nature of this interaction is unknown although in rodent skin (for example, see [70,71]).

The flattened end of the elongated hair peg molds into a bulb, carrying internally the matrix cells that line the concavity of the bulb and the associated mesenchymal cells that become the dermal papilla. The sheath encloses the entire structure, internalizing and confining the cells of the dermal papilla to an environment surrounded almost entirely by follicle epithelial cells (Fig 9). This protected matrix-dermal papilla unit is supplied in the adult by capillaries and nerve fibers but neither is seen to intrude during development. Ultrastructural studies of the bulb reveal a stratification of mesenchymal cells that appear to differ in synthetic activity. Tightly packed cells surrounded by little or fibrous or filamentous matrix form an innermost mesenchymal layer that is closely associated with the basement membrane beneath the follicle matrix (Fig 10a). The more peripheral fibroblasts of the dermal papilla are elongated and continuous with the sheath cells laterally (Fig 10b). An intermediate layer of mesenchymal cells has properties transitional between the compact and the peripheral cells. Immunolabeling of the dermal papilla reveals an enhancement of basement membrane-like matrix molecules (LN, HSPG, entactin/nidogen, type IV collagen). Interstitial fibrous proteins are expressed in minor quantities [41], although the cells in both outer two zones are surrounded by small bundles of collagen fibrils and are faintly immunopositive when reacted with antibodies against collagen types I, III, V, and VI, and fibrillin. The immunostaining data support the concept of functional as well as morphologic differences among dermal papilla cells. Immunostaining for HSPG reveals a central core of immunopositive cells in the dermal papilla and in the basement membrane; the mesenchymal cells immediately adjacent to the matrix and the sheath cells are not immunopositive for this molecule. The mesenchymal cells and extracellular matrix of the dermal papilla are also strongly immunoreactive with antibodies against N-CAM, TN, both PDGF-Bs, and p75. The sheath lacks TN, thus leaving only the dermal papilla immunoreactive with this marker. Labeling for the interstitial collagen and fibrillin outlines the orientation of the matrix within the sheath.

Figure 8. Schematic drawing of the Bulbous Hair Peg stage illustrating the regions of the differentiated follicle including the bulb, matrix, developing hair, and layers of the root sheaths. The sebaceous gland and duct, bulge, and hair canal are also shown. Note the mesenchyme enclosed within the bulge forming the dermal papilla. The cells of the dermal papilla are continuous with those of the follicle sheath.
Table III. Comparison of the Expression of Selected Growth Factors and Receptors, Cell Adhesion Molecules, Structural Proteins, and Matrix Molecules by Cells of the Follicular and Interfollicular Epithelium, the Basement Membrane Between the Two Regions, and the Related Mesenchymal Tissue

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Coordinate with the morphologic appearance of the concentric layers within the follicle is the expression of markers associated with keratinization: hair keratins in the fiber, trichohyalin in the root sheath, and cornified cell envelope proteins and transglutaminase in the inner root sheath. Epidermal keratins (K5/K14), as well as K6, K16, and 17, continue to be expressed in the ORS, sebaceous gland, and infundibulum of the follicle [17,18]. The infundibulum is the only site where keratin expression is evident to that of the epidermis proper. The 40-kDa keratin (K19) is evident only in the bulge and the ORS of the follicle distal to the bulge [13], the region of the follicle that atrophies and regrows during the hair cycle.

We have developed a suspension organ culture system (SOC) [15] that permits long term maintenance, growth, morphogenesis, and differentiation of explants of human embryonic and early (60-85 d) fetal skin. The samples form completely enclosed spheres with the epidermis outward 3 dafter tissue is explanted. There is no evidence of the site of closure after 1 week in culture (Fig 11). Ultrastructural studies of samples that have been maintained for 30 d reveal an intact dermal-epidermal junction and persistent attachment structures (hemidesmosomes and anchoring fibrils). The dermal cells and matrix are maintained and the epidermis undergoes differentiation. Filament-associated keratohyalin granules, a cornified cell envelope, and lamellar granules form in upper spinous and granular cells. Melanocytes that were present in the epidermis at the time the tissue was explanted become highly dendritic, synthesize abundant melanin, and transfer melanosome to the keratinocytes.

The culture system also permits several aspects of follicle morphogenesis to occur depending upon the age of the tissue explanted.

Figure 9. Light (*a*) and electron (*b*) micrographs of the bulb of a 140-d EGA bulbous hair peg. The DAPI fluorescent nuclear stain demarcates the layers of cells in the follicle by the orientation of their nuclei and shows the density of mesenchymal cells clustered in the dermal papilla (DP). The electron micrograph shows the keratinocytes in different layers of the follicle from the outermost layer of the outer root sheath (ORS) centrally. Some keratinization of the inner root sheath (IRS) is evident. The matrix of the bulb is proximal to the basement membrane and in juxtaposition to the mesenchymal cells of the dermal papilla. (*a*) magnification X215; (*b*) magnification X700.

Figure 10. Electron micrographs of the dermal papilla cells showing the apparent absence of fibrillar extracellular matrix in regions proximal to the matrix (M) (*a*). Fibrillar collagen (arrow) surrounds the mesenchymal cells located proximal to and contiguous with the follicle sheath (*b*). (*a*) magnification X1512; (*b*) magnification X2295.
yet samples of skin in this age range initiate hair germs in a regular pattern, and carry morphogenesis to the hair peg stage (Fig. 11). Are they required for induction to occur? We suspect that induction cannot occur in the embryonic period. They are also missing from older explants that do demonstrate follicle conditions do not appear to be sufficient for follicle morphogenesis to begin in the germ. Aggregation of mesenchymal cells may require information from aggregation, or a systemic influence outside of the tissue may be necessary. The skin increases in complexity as development progresses and exceeds to completion, but requires more complex conditions and an artificial matrix may alter the polarity and spatial relationships of keratinocytes and prevent normal elongation, or the dermis may not provide adequate support. We have been stressing, from perhaps a narrow perspective, that the immediate mesenchyme of the follicle is the important environment. However, studies of tissue recombinants with dermis and other connective tissue matrices, including artificial matrices, have shown a need for matrix organization in appendage morphogenesis.

Appendages may fail to form in explanted embryonic skin because a) molecules considered "morphogens," perhaps inducers, are not expressed by either the epithelial or mesenchymal cells (or both) to start the process; b) the "right" cells (perhaps a specific collection of mesenchymal cells) may be missing; c) the epidermis may need to stratify in vitro; d) the cultures may not grow long enough to permit formation of the follicle; and/or e) induction may need to occur in vitro prior to explantation to provide some critical element(s) that cannot be accommodated in culture (and may be unrelated to any of the molecules whose expression we have examined).

To determine whether the "prefollicle" conditions are sustained in the epidermis and compact mesenchyme during the first 2 weeks in culture we immunostained SOCs from each of the three age groups at 7 and 14 d in culture. The expression of keratins, type VII collagen, and p75 were the same as in vivo. There were no dermal condensations in any of the samples signifying follicle initiation. Aggregation of mesenchymal cells may require information from the epidermis that is not provided, the dermal cells may be unable to synthesize molecules (e.g., epimorphin [5]) that are needed for aggregation, or a systemic influence outside of the tissue may be necessary. We know that the epidermis is stratified when the follicle germ develops in vivo. Stratification of embryonic epidermis occurs rapidly in culture and the cells express the markers characteristic of keratinocyte differentiation once they stratify. Nonetheless, these conditions do not appear to be sufficient for follicle morphogenesis to begin in vitro. Intact nerves and vessels are missing of course, but they are also missing from older explants that do demonstrate follicle formation. Are they required for induction to occur? We suspect that induction cannot occur in vitro and does not occur in vivo during the embryonic period.

Early fetal skin does not have follicle primordia at 65 - 75 d EGA, yet samples of skin in this age range initiate hair germs in vitro, in a regular pattern, and carry morphogenesis to the hair peg stage (Fig. 12). The experiments are highly reproducible as long as the dermis survives [15]. The structure of the early germs, later germs, and early hair pegs is comparable to the counterpart structures in vivo with the following exceptions: the dermal condensation seems to include a greater number of mesenchymal cells and the epidermal cells of the germ may become more densely packed and less organized.

The success of tissue in this age group in initiating and advancing follicle formation may indicate that induction occurred in vivo before the tissue was explanted and that the messages exchanged during that time were instructive and perhaps sufficient to begin and sustain the early stages of follicle morphogenesis. Alternatively, it is possible that induction can occur in vitro during the 21 - 30-d period before the aggregation of mesenchymal cells and clustering of epithelial cells are observed in the explants. The similarity of expression of phenotypic markers in the hair germ and hair peg in vivo suggests that "new directions" may not be required. This does not support a conclusion, however, that no further directions are needed to continue development after induction and initiation; it is likely that the exchange of messages continues throughout these two stages of development in vitro and that they may be instructive, especially at the onset of differentiation. Based on the observation that follicles present in fetal skin at the time of explantation degenerate rapidly in vitro, we suspect that the culture system supports early stages of follicle morphogenesis in the 65 - 75-d fetal skin in more than a passive manner.

Follicle development in vitro does not proceed beyond the early hair peg stage. Elongation is abrogated and differentiation fails to occur. The structure is maintained variously in this halted stage of development. Further development may not proceed because critical factors are exhausted or new factors that are required may not be synthesized or available in vitro. Abnormalities in the follicle basement membrane may alter the polarity and spatial relationships of keratinocytes and prevent normal elongation, or the dermis may not provide adequate support. We have been stressing, from perhaps a narrow perspective, that the immediate mesenchyme of the follicle is the important environment. However, studies of tissue recombinants with dermis and other connective tissue matrices, including artificial matrices, have shown a need for matrix organization in appendage morphogenesis.

Hair germs and hair pegs present in older fetal skin at the time the tissue is explanted may continue to develop for a short period, then degenerate. Failure of the SOC system to support follicles initiated in vitro suggests that the process once initiated does not simply proceed to completion, but requires more complex conditions and an environment beyond that of interacting components themselves. The skin increases in complexity as development progresses and perhaps becomes more restricted in the processes it can support.

![Figure 11](image1.png) **Figure 11.** Samples of 67-(a) and 70-(b) d EGA fetal skin that have been grown in suspension organ culture (SOC) for 32 d (a) and for 72 h after explantation. The two SOCs in (a) demonstrate the large size and the spherical shape of the skin samples after this period in culture. The SOC in (b) has been photographed in the scanning electron microscope to show how rapidly the sample of skin closes to form a spherule. The surface of the SOC is the pristim. (a) magnification × 10; (b) magnification × 425.

![Figure 12](image2.png) **Figure 12.** Fetal skin between the ages of 65 - 70 d EGA grown in suspension organ culture for 28 d shows the formation of hair germs. Magnification × 125.
The three age groups identified—one that fails to support follicle morphogenesis, another in which follicle morphogenesis is activated, and a third in which follicles already formed are degenerated—allow us to focus on these time periods in an attempt to identify factors that can "correct" the conditions and permit morphogenesis to be initiated and/or to proceed.

CONCLUSIONS

We have demonstrated in the developing follicle the developmental expression of molecules that have been shown in other systems to have morphogenetic potential for epithelial and mesenchymal cells. Some markers appear to be associated uniquely with either the epithelial or mesenchymal cells or the basement membrane zone of the developing follicle (PDGFRs, p75, N-CAM, TN, EGFBR, collagens I, III, V, and VI, and C4-SPG), whereas others (e.g., structural proteins of keratinocytes and certain extracellular matrix molecules) most likely reflect the outcome of the interactions and status of development. The molecules we have discussed represent only a fraction of the markers that have been investigated by immunohistochemistry and in situ hybridization by others studying different kinds of follicles (vibrissae and pelage) in nonhuman tissue (e.g., rodent and sheep) and analogous appendages (e.g., teeth, feathers). Many of these studies, however, have concentrated on a specific interaction at a given stage of development, few investigators have followed the entire process of follicle morphogenesis from initiation through differentiation. The in vivo studies do not permit conclusions about the reciprocal events that must occur in order to initiate and sustain follicle development.

Many of the molecules we have considered are "peptide regulatory factors," suggested by Slack [73] to be "inducing factors"; the cells that bear receptors for these factors are thought to be "competent" to respond. "Commitment" occurs when the interaction activates a particular signal transduction pathway. We know something about the environment surrounding the interaction and a few characteristics of the modified phenotype of the epithelial and mesenchymal cells that participate, compared with their counterpart cells in the interfollicular epidermis and the dermis, but whether the phenotypic properties reflect induction or may have promoted induction remains to be determined. Induction remains an event we don't "see," which occurs at a time we can't specifically identify, through the exchange of messages we haven't deciphered, and in a sequence not yet defined.

Answers to some of our questions about follicle morphogenesis may be forthcoming from experimental studies that can be conducted using human and animal skin in vitro and in experimental animals in vivo. We have described an organ culture system that supports the growth and development of human fetal skin for extended periods and thus an opportunity to investigate the nature of the interactions between epithelial and mesenchymal cells during early stages of follicle morphogenesis. To our knowledge, it is the only system that allows for the initiation of follicles in human skin in vitro using skin that shows no evidence of follicles at the time of explant. At present, the SOC system has been used to grow human fetal skin in the 65-75-d age range for as long as 12 weeks, during which time relatively normal progression of development and differentiation occurs; in appropriately aged skin explants, follicle morphogenesis is initiated in vitro [15]. The large size and often cyst-like nature of the SOCs allows cells and molecules such as growth factors, blocking antibodies, inhibitory oligonucleotides, matrix-stimulating molecules, etc. to be injected into the cultures. Fluid can be removed from the skin SOCs to assay for the synthesis of growth factors and other molecules produced by the tissues. The unattached state of the tissue allows it to be modified by natural factors (conditioned medium) produced by keratinocytes/fibroblasts and/or other cells cultured in the same dishes. Use of this system may help us meet the challenges of learning more about skin development and follicle morphogenesis on a more mechanistic basis.

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