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HOX Homeobox Genes Exhibit Spatial and Temporal Changes in Expression During Human Skin Development

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The spatial and temporal deployment of *HOX* homeobox genes along the spinal axis and in limb buds during fetal development is a key program in embryonic pattern formation. Although we have previously reported that several of the *HOX* homeobox genes are expressed during murine skin development, there is no information about developmental expression of *HOX* genes in human skin. We have now used reverse transcriptase polymerase chain reaction, in conjunction with a set of degenerate oligonucleotide primers, to identify a subset of *HOX* genes that are expressed during human fetal skin development. *In situ* hybridization analyses demonstrated that there were temporal and spatial shifts in expression of these genes. Strong *HOXA4* expression was detected in the basal cell layers of 10 wk fetal epidermis and throughout the epidermis and dermis of 17 wk skin, whereas weak signal was present in the granular layer of newborn and adult skin. The expression patterns of *HOXA5* and *HOXA7* were similar, but their expression was weaker. *In situ* hybridization analysis also revealed strong *HOXC4* and weaker *HOXB7* expression throughout fetal development, whereas *HOXB4* was expressed at barely detectable levels. Differential *HOX* gene expression was also observed in developing hair follicles, and sebaceous and sweat glands. None of the *HOX* genes examined were detected in the adult dermis. *Key words: dermis/epidermis/gene regulation/homeodomain. J Invest Dermatol 110:110–115, 1998*

Human skin is comprised of a self-renewing epidermis derived from ectodermal tissue, and an underlying dermis, which is of mesodermal origin. There is increasing evidence that BMP-4 (Nohno *et al*, 1995a), and other signaling molecules such as sonic hedgehog (Nohno *et al*, 1995b), TGF-α (Luetteke *et al*, 1993; Mann *et al*, 1993), and EGF (Miettinen *et al*, 1995; Murillas *et al*, 1995), play important inductive roles in epithelial-mesenchymal signaling during skin development and hair formation. It has been hypothesized that a set of transcription factors must mediate the effects of these putative morphogens to regulate skin-specific gene transcription. In this regard, the Lef-1 transcription factor has been shown to play a role in whisker and hair formation (van Genderen *et al*, 1994; Zhou *et al*, 1995); however, the molecular mechanisms that regulate skin and hair development remain largely unknown.

The *HOX* homeobox genes appear to be candidates for the regulation of skin development. These master developmental regulatory genes encode proteins that contain a conserved 60 amino acid, DNA binding homeodomain and function as transcription factors (Gehring *et al*, 1994). The 39 human homeobox genes are arrayed in four parallel chromosomal clusters such that individual genes from each loci (e.g., *HOXA4*, *HOXB4*, *HOXC4*, and *HOXD4*) show a high degree of homology within their putative homeodomains. The HOX homeodomain proteins appear to regulate the formation of multiple structures along various body axes such as the spinal cord, and limbs (Krumlauf, 1994). We (Detmer *et al*, 1993; Mathews *et al*, 1993) and others (Bieberich *et al*, 1991; Reiger *et al*, 1994) have previously detected expression of several of the *Hox* genes, particularly those in the *Hox-b* cluster, in fetal and adult murine skin. In addition, there have been several reports of spatially restricted expression of *Hox* genes in developing murine (Bieberich *et al*, 1991; Kanzler *et al*, 1994) and chicken skin (Chuong *et al*, 1990). Based on these studies and the well-recognized role of homeodomain proteins in development, Scott and Goldsmith proposed that the *HOX* homeobox genes play a major regulatory role in human skin development (Scott and Goldsmith, 1993). Until now, however, there have not been studies on the spatial and temporal expression of *HOX* genes in developing human skin. We report here the use of a reverse transcriptase polymerase chain reaction (RT-PCR) based approach to identify which of the 39 human *HOX* genes are expressed in first and second trimester human fetal skin and which are expressed in adult skin. Only a small subset of *HOX* genes were detected using this method. *In situ* hybridization analysis revealed spatial and temporal changes in the expression of these genes within both the developing epidermis and the dermis, as well as in hair follicles.

MATERIALS AND METHODS

Tissue samples Tissue samples were obtained under NIH guidelines and after approval of the University of California San Francisco Committee on Human Research. Tissue was fixed in 4% paraformaldehyde for 4 h, dehydrated in graded ethanol, and embedded in paraffin. For some tissues, half was frozen in solution D (see below) for subsequent RNA isolation. Following informed consent newborn and adult skin samples were obtained from the margins of specimens from elective surgery.

Manuscript received September 16, 1996; revised August 20, 1997; accepted for publication October 14, 1997.

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Abbreviation: RT-PCR, reverse transcriptase polymerase chain reaction.

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Dermo–epidermal separation Five samples of second trimester fetal scalp skin (18–22 wk) and five samples of adult skin (50–65 y) were subjected to dermal/epidermal separation by exposure of full thickness skin samples to dry heat (60°C for 60 s) (Holleran *et al*, 1992). The epithelium was then scraped off the dermis and placed in a separate container for RNA isolation.

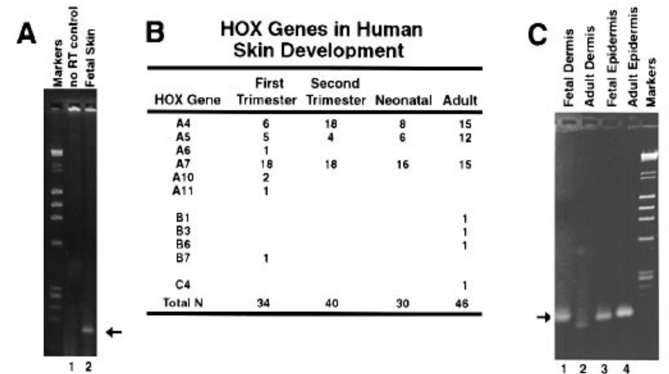
RNA isolation Total RNA was isolated from fetal, neonatal, and adult skin using the guanidinium thiocyanate method (Chomczynski *et al*, 1987). Skin from the scalp, back, or limbs was homogenized in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1% 2-mercaptoethanol), and RNA was separated from DNA and protein by extraction with acid phenol:chloroform:isoamyl alcohol (50:49:1). RNA concentrations were estimated by the optical density at 260 nM, and RNA was stored in 70% ethanol at –70°C.

RT-PCR RT-PCR was used to survey for *HOX* gene expression in each sample. We used a protocol that included two polymerase chain reaction (PCR) amplifications (Sauvageau *et al*, 1994). First, amplified cDNA representing total mRNA was synthesized from each tissue RNA. A subsequent PCR amplification using degenerate primers designed against all 39[1] *HOX* genes permitted their identification by cloning and sequencing of the PCR reaction products. The first strand cDNA was synthesized by adding total RNA (1 μg) to 10×reverse transcriptase buffer, dNTP, Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD), and an oligo dT primer: (5′-CATGTCGTCCAGGCCG-CTCTGGACAAAATATGAATTC $(T)_{21}$–3′). Following reverse transcription and phenol/chloroform extraction, a 5′ polyA tail was added to the cDNA using terminal transferase. The same oligo dT primer was then re-added to the solution (1 μg per μl) and PCR was performed (94°C, 2 min; 55°C, 3 min; 72°C, 10 min) for 35 cycles.

The amplified cDNA mixtures were screened using a set of degenerate *HOX* gene primers derived from two conserved regions of the homeobox, separated by 118 nt: (5′-GA (G/A) (T/C)T (G/A/T)GA (A/G) (C/A/G) (G/A) (G/A/C)GA (A/G)T (A/T) (T/C)-3′) and (5′-NC (G/T) (A/G)TT (T/C)TG (A/G)AACCANA (C/T)-3′). A Hot Wax PCR kit (10 × buffer pH = 10.0 and 1.5 MgCl beads) (Invitrogen, La Jolla, CA) was used to maximize the PCR reaction (94°C, 2 min; 48°C, 2.5 min; and 72°C, 3 min) for 32 cycles. This procedure produced a 118 bp band that was detected on a 2% agarose gel (FMC, Rockland, ME) and that contained amplified products reflecting the homeobox gene transcripts present in each tissue sample.

In order to identify the various homeobox cDNA derived from each sample, the 118 bp DNA fragments were cut from the gels, purified (Bio 101, Vista, CA), and ligated into the T/A PcRII vector (Invitrogen). Insert-containing clones were sequenced using ^{35}S-ATP and the Sanger dideoxy method (Promega, Madison, WI). Sequences were identified by comparison to the known 38 human *HOX* genes (Genbank) using GCG-8 software (Genetics Computer Group, Madison, WI).

RNA template labeling, probe preparation, and *in situ* hybridization Templates for human *HOXA4*, *HOXA5*, *HOXA7*, *HOXB4*, *HOXB7*, and *HOXC4* were constructed by subcloning 3′ nonhomeobox fragments in Bluescript SK$^+$ (Stratagene, La Jolla, CA). Linearized plasmids (1 μg) were used to synthesize digoxigenin-labeled sense and anti-sense RNA probes (Boehringer, Indianapolis, IN). RNA *in situ* hybridization was performed as described (Mathews *et al*, 1993) with modifications to use digoxigenin labeled-probes (Wilkinson, 1992). Sections were deparaffinized in three changes of Americlear (Baxter Diagnostics, Deerfield, IL) and re-hydrated through graded ethanol. Following washing, sections were digested with proteinase K (10 μg per ml) for 30 min at 37°C, rinsed in physiologically buffered saline, washed in 0.1 M triethanolamine with 0.25% acetic anhydride, prehybridized in 50% formamide, 2 × sodium citrate/chloride buffer for 1 h at 37°C, and then allowed to air dry. The digoxigenin-labeled probes were diluted in hybridization solution (2 × sodium citrate/chloride buffer, 12.5 × Denhardt's solution, 50% formamide, 0.5% sodium dodecyl sulfate, 0.25 μg salmon sperm per ml, 0.5% sodium pyrophosphate, 10 mM TrisHCl, pH 7.4), applied to each section, which was then covered with parafilm and incubated overnight at 42°C. Sections were washed, treated with RNAse A (20 μg per ml), washed in a series of increasing



HOX Gene	First Trimester	Second Trimester	Neonatal	Adult
A4	6	18	8	15
A5	5	4	6	12
A6	1			
A7	18	18	16	15
A10	2			
A11	1			
B1				1
B3				1
B6				1
B7	1			
C4				1
Total N	34	40	30	46

Figure 1. HOX homeobox genes are expressed in developing skin. (*A*) PCR analysis, using degenerate primers designed to amplify a region of the homeobox from any of the known 38 human *HOX* genes, was used to detect *HOX* transcripts in whole second trimester fetal skin. Following an initial RT-PCR amplification using oligo dT primers to generate an amplified cDNA, a second round of PCR was performed with the degenerate primers to generate a 118 bp DNA fragment (→, *lane 2*) reflecting the presence of *HOX* transcripts. *Lane 1* is an identical sample in which reverse transcriptase was omitted from the initial cDNA synthesis step. (*B*) Identification of *HOX* gene transcripts in whole skin samples. The 118 bp PCR products from each skin sample, prepared as shown in (*A*), were cloned and sequenced, and comparison with published sequences permitted identification of *HOX* transcripts. The values shown represent the number of clones that matched a given *HOX* gene sequence. Expression of the other 27 known human *HOX* homeobox genes was not detected. (*C*) *HOX* genes were not detected in adult dermis. The degenerate primer PCR method was also used to amplify *HOX* transcripts from fetal and adult skin samples that have been split into epidermis and dermis. Appropriately sized PCR products were detected in both fetal and adult epidermis (→, *lanes 3* and *4*), as well as in fetal dermis (*lane 1*); however, a 118 bp band was not detected in the adult dermis (*lane 2*). Cloning and sequencing of the smeared PCR products from the adult dermis confirmed that no *HOX* transcripts could be detected. In contrast, the PCR bands for the fetal and adult epidermis and the fetal dermis all gave data similar to those reported in (*B*).

stringency solutions, and rinsed in buffer (100 mM TrisHCl, pH 7.5, 150 mM NaCl). Sections were incubated (1 h) in anti-digoxigenin-horseradish peroxidase antibody (1:500) in buffer [1% non-fat dried milk, 0.5% fish skin gelatin, 10 mM 500 mM NaCl, 0.1% Tween 20, TrisHCl (pH 7.6)], then incubated with biotinylated-tyramide (15 min), followed by a streptavidin-peroxidase (15 min) (Dako, Carpinteria, CA). Signals were visualized with DAB chromogen substrate (5 min). For some *HOXA5* experiments, the color development time was 25 min. To detect *HOXA7* signals, color development was for 25 min and a DAB color enhancer (Innovex Biosciences, Richmond, CA) was added to the reaction. Some sections were counter-stained with hematoxylin to visualize tissue morphology. Photographs were taken with a Nikon Microphot FX photomicroscope and digitized using a Nikon Coolscan slide scanner. Composite images were created with Adobe Photoshop, and printed on a Kodak dye sublimation printer. Except for background color adjustment, no other image processing was performed.

RESULTS

A subset of HOX homeobox genes is expressed in developing human fetal skin We first used an RT-PCR/sequence analysis to identify *HOX* homeobox genes expressed in human fetal and adult scalp skin. With this method, an initial RT-PCR-amplified cDNA library representing the total mRNA of each tissue sample was constructed. This cDNA was then subjected to a second round of PCR amplification using a set of degenerate PCR primers, derived from conserved portions of the homeobox region, which were designed to amplify all of the human *HOX* genes. **Figure 1*A*** shows a representative experiment in which the expected 118 bp PCR product band was detected in RNA isolated from whole second trimester fetal scalp skin. The 118 bp PCR product band derived from each skin sample was excised from the gel, cloned, and sequenced. Clones containing *HOX* gene transcripts were identified by comparison with published sequences of the 38 known *HOX* genes.[1] When the PCR products were cloned and identified, the majority of the *HOX* gene transcripts detected in fetal skin (**Fig 1*B***) belonged to the *HOX A*

[1]At the start of this project, 38 *HOX* genes had been described. As part of this work, an additional human *HOX* gene was identified in developing dermis. The new gene, *HOXB13*, appears to be differentially regulated in scarless fetal wound healing and is the subject of a submitted manuscript (Stelnicki EJ, Arbeit J, Cass DL, Saner C, Harrison M, Largman C: Changes in expression of the human homeobox genes PRX-2 and HOXB13 are associated with scarless fetal wound healing. Manuscript submitted). This gene was independently isolated by Zeltser *et al* (1996) during these studies. The degenerate primers used contained a match for the *HOXB13* homeobox.

cluster. Of these, *HOXA4*, *HOXA5*, and *HOXA7* appeared to be predominant. Transcripts for *HOXA6*, *HOXA10*, *HOXA11*, and *HOXB7* were also detected with lower frequency in whole fetal skin. It must be emphasized that it is not possible to use these data to compare the relative levels of expression between time points. Like the fetal tissue, the majority of *HOX* gene transcripts detected in the human neonatal scalp were *HOXA4*, *HOXA5*, and *HOXA7* (**Fig 1*B***). This same trend continued in the adult tissue, with the additional detection of several members of the *HOX-B* cluster (*HOXB1*, *HOXB3*, and *HOXB6*) and one member of the *HOX-C* locus (*HOXC4*) (**Fig 1*B***). The RT-PCR analysis was repeated on RNA isolated from back and arm skin from first and second trimester embryos as well as from newborn foreskin. These experiments revealed the same pattern in which *HOXA4*, *HOXA5*, and *HOXA7* were the major transcripts detected (data not shown).

HOX gene transcripts are not detected in adult dermis by RT-PCR A similar RT-PCR analysis was performed on fetal and adult skin samples that were split into epidermis and dermis. Although the expected 118 bp PCR band representing amplification of *HOX* transcripts was detected in fetal epidermis and dermis, as well as in adult epidermis, there was no apparent *HOX* gene expression in adult dermis as reflected by the absence of the appropriate size PCR product in multiple (N = 5) samples (**Fig 1*C***, *lane 2*). When the PCR bands from the fetal epidermis and dermis and the adult epidermis were cloned and sequenced, data similar to those shown in **Fig 1*B*** were obtained (not shown). As anticipated by the lack of a 118 bp RT-PCR band, cloning of the PCR products from adult dermis did not yield identifiable *HOX* gene sequences.

HOXA4 is expressed in a temporal and spatial pattern during epidermal development In order to obtain spatial localization of *HOX* gene transcripts, we used *in situ* hybridization to analyze expression during skin development. We analyzed *HOX* gene expression in skin from the scalp, back, and arm from first trimester (10 wk estimated gestational age), early second trimester (14 wk estimated gestational age), mid-second trimester (17 wk estimated gestational age), and late second trimester (21 wk estimated gestational age) embryos, as well as from normal newborn (4 mo old) and adult skin samples. Because *HOXA4*, *HOXA5*, and *HOXA7* appeared from the RT-PCR data to be strongly expressed in developing skin, we first examined expression of these genes at each developmental time point (**Fig 2**). Because the signals for *HOXA4* were quite strong, these sections were counter-stained with hematoxylin to visualize tissue morphology (**Fig 2*A–F***). Weaker *HOXA5* (**Fig 2*G–L***) and *HOXA7* (**Fig 2*M–R***) signals were visualized on adjacent tissue sections without counter-staining. In order to be able to compare *HOXA4* expression with that of the other genes, a subset of sections was also examined for *HOXA4* signals in the absence of counter-staining (**Fig 3*A–C***). *HOXA4* was predominantly expressed in the basal cell layer, and was localized to the lower layers of the developing epidermis in the early (10 wk) embryonic skin (**Figs 2*A*, 3*A***). Control slides in which tissues were hybridized with a sense RNA probe were negative (see **Fig 3*R***). Cells of the periderm (present at 10 and 14 wk) did not express *HOXA4*, or any other *HOX* gene studied. *HOXA4* expression appeared to gradually spread throughout the epidermis from 10 to 17 wk of development until by mid-second trimester, the signal was detected in the entire epidermis, except the stratum corneum (**Figs 2*B*,*C*, 3*B***). It should be noted that the stratum corneum was uniformly negative at each developmental stage for every probe studied. By late second trimester *HOXA4* expression was less prevalent in the basal cell layer but was still detected throughout the suprabasal epidermis (**Figs 2*D*, 3*C***). *HOXA4* expression was downregulated further in the basal and stratum spinosum layers of the newborn (**Fig 2*E***) and adult skin (**Fig 2*F***), where expression appeared to be localized to the upper granular layers of the epidermis. The specimens shown in **Fig 2(*A–F*)** were all from scalp except the 14 wk sample (**Fig 2*B***) that was from the back. *In situ* hybridization analysis of *HOXA4* expression in the corresponding series of back or arm skin samples showed essentially identical temporal and spatial changes throughout development (data not shown). In addition, the relative expression levels of *HOXA4*, as well as those of the other *HOX* genes described below, did not vary substantially between these three tissues during development.

HOXA4* expression is temporally regulated in the dermis during skin development** *HOXA4* expression appeared to be virtually absent in the dermis of first trimester (week 10, **Figs 2*A*, 3*A) and early second trimester (week 14, **Fig 2*B***) fetal skin; however, by mid-second trimester (week 17, **Figs 2*C*, 3*B***) *HOXA4* expression was seen in dermal fibroblasts. Weak expression persisted at late second trimester dermis (week 21, **Figs 2*D*, 3*C***), but was absent in newborn (**Fig 2*E***) and adult (**Fig 2*F***) dermis. Fibroblasts in the dermal papillae did not express HOXA4 or other *HOX* genes studied (**Figs 2*B–D*, 3*M***, see also the section on hair follicle development below).

HOXA5* and *HOXA7* are expressed in similar patterns but at lower levels than *HOXA4* in developing skin** *In situ* hybridization experiments revealed that *HOXA5* (**Fig 2*G–L) was detected at lower levels but in a similar pattern to *HOXA4*, whereas initial experiments with a *HOXA7* probe gave very weak signals. We used increased color development time and an additional amplification step in order to localize expression of the *HOXA7* gene (**Fig 2*M–R***), which appeared to follow similar temporal and spatial patterns to those observed for *HOXA4*. Thus, expression appeared to move from the basal layers in early epidermal development towards the outer epidermis in newborn and adult skin. Weak expression of *HOXA5* and *HOXA7* was detected in the fetal dermis during the second trimester of fetal development (**Fig 2*I–O***), but no expression of these genes was observed in newborn (**Fig 2*K*,Q**) or adult dermis (**Fig 2*L*,*R***).

HOXB4*, *HOXB7*, and *HOXC4* are variably expressed in developing skin** We also used *in situ* hybridization to assess the expression patterns of *HOX* genes detected at lower levels by the RT-PCR method (*HOXB7* and *HOXC4*), as well as a gene not detected by this procedure (*HOXB4*). *HOXC4* was expressed in similar temporal and spatial patterns to those observed for *HOXA4* (compare **Fig 3*J–L with **Fig 3*A–C***), whereas *HOXB4* was barely detectable (compare **Fig 3*D–F*** with **3*R***). *HOXB7* was weakly expressed in the basal and suprabasal layers of the 10 wk old skin (**Fig 3*G***), was downregulated in early second trimester skin (**Fig 3*H***), and appeared to be upregulated throughout the epidermis in 21 wk skin (**Fig 3*I***). Expression of each f these genes was downregulated and restricted to the upper epidermis in newborn and adult skin (data not shown). Essentially identical results were obtained using skin from either the back or the arm (not shown).

Differential *HOX* gene expression during hair follicle, sweat gland, and sebaceous gland development As seen in **Fig 2*B***, early hair follicle formation was observed in the 14 wk skin. At this time, none of the *HOX* genes studied could be detected in the prospective bud (**Fig 2*B*,*H*,*N***, and data not shown); however, by 17 wk, clear expression of *HOXA4* (**Figs 2*C*, 3*B***) and *HOXA5* (**Fig 2*I***) was detected in developing follicles, whereas *HOXC4* expression was weak but noticeable (**Fig 3*K***). None of the other *HOX* genes examined were detected in the developing hair follicles of the 17 wk skin. By 21 wk, signals for *HOXB7* in the maturing hair follicles (**Fig 3*I***) were approximately equal to those observed for *HOXA4* (**Figs 2*D*, 3*C*, and 3*M***) or *HOXC4* (**Figs 3*L* and 3Q**), whereas expression of the other genes was not detected. In newborn skin, the mature follicles showed expression of *HOXA4* (**Figs 3*M* and 3O**), *HOXA5* (**Fig 3*P***), and *HOXA7* (**Fig 2Q**). More importantly, these *HOX* genes appeared to be expressed in somewhat different spatial patterns in the mature hair follicles in 21 wk and 4 mo skin. Thus *HOXA4* (compare **Figs 3*M*** and **3*N***, and see **Fig 3O**), *HOXA7* (**Fig 2Q**), and *HOXC4* (**Fig 3Q**) were expressed in all the epidermal layers of the hair follicle but not in the melanocytes or in the dermal papillae. In contrast, *HOXA5* expression was restricted to the inner root sheath (**Fig 3*P***). Only extremely weak *HOX* gene expression was detected in the hair follicles within the adult skin (data not shown). In addition to prevalent expression in hair follicles, *HOXA4* was clearly also expressed in sweat glands in newborn skin (**Fig 3O**) and adult skin (data not shown). Expression of the other *HOX* genes studied was not detected in sweat glands at any stage of development. *HOXA4* was also clearly detected

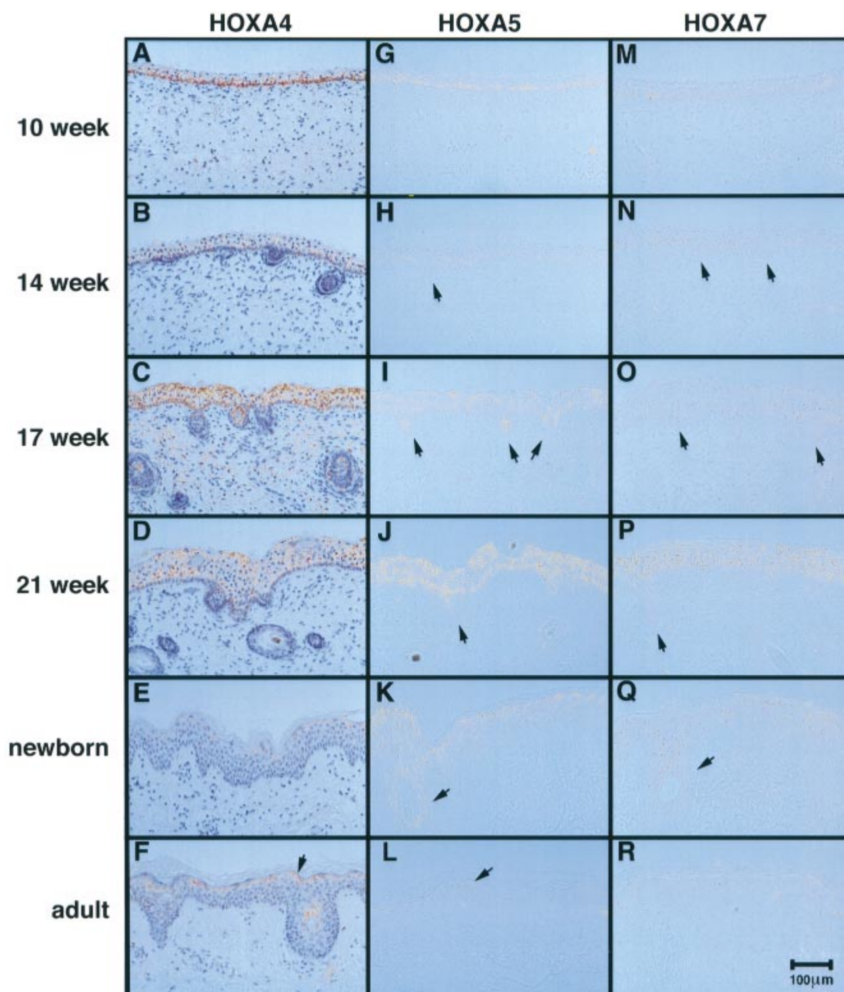


Figure 2. *HOXA4*, *HOXA5*, and *HOXA7* genes are expressed in spatial and temporal patterns during skin development. *In situ* hybridization analysis was used to detect expression of *HOXA4* (*A–F*), *HOXA5* (*G–L*), and *HOXA7* (*M–R*) in serial sections from 10 wk scalp (*A*, *G*, and *M*), 14 wk back (*B*, *H*, and *N*), 17 wk scalp (*C*, *I*, and *O*), 21 wk scalp (*D*, *J*, and *P*), newborn scalp (*E*, *K*, and *Q*), and adult scalp (*F*, *L*, and *R*). Parts (*A*)–(*F*) were counterstained with hematoxylin to visualize tissue morphology. Parts (*G*)–(*L*) and (*M*)–(*Q*) represent adjacent sections to those stained in (*A*)–(*F*). Digoxigenin-labeled RNA probes were hybridized with tissue sections, and a tyramide amplification and peroxidase detection was used to visualize *HOX* gene expression as described in *Materials and Methods*. Whereas detection of *HOXA4* was visualized with 5 min color development, *HOXA5* was visualized at 25 min of color development, and an additional color intensification was also applied for *HOXA7* signal detection. For comparison, sections hybridized with a control sense RNA probe showed no signal (see **Fig 3*R***). All the → point to developing hair follicles except the → in (*L*), which denotes the weak expression of *HOXA5* in the granular layer of the adult skin. *Scale bar*, 100 μm.

in newborn and adult sebaceous glands, whereas expression of the other *HOX* genes was not detected (data not shown).

DISCUSSION

The *HOX* homeobox genes appear to be important developmental regulatory genes in animals as diverse as *Drosophila* to man (Krumlauf, 1992). These 39 genes encode transcription factors that appear to both activate and repress embryonic gene expression. Given these roles in other tissues, we and others have anticipated that the *HOX* genes might play roles in skin development (Scott and Goldsmith, 1993). Using a PCR-based screening method, the most commonly detected *HOX*-gene transcripts in human skin were *HOXA4*, *HOXA5*, and *HOXA7*. In addition, low level expression of several additional *HOXA* and *HOXB* genes as well as the *HOXC4* gene were detected by PCR in the fetal or adult skin samples. *In situ* hybridization analysis confirmed that *HOXA4* was expressed at high levels in developing skin. In addition, *HOXC4* was expressed at high levels in developing skin whereas *HOXA5*, *HOXA7*, and *HOXB7* were expressed at lower levels, and *HOXB4* was barely detected. Our observations confirm previous studies that have described detection of *HOXA7* (Thomas *et al*, 1989) and *HOXB7* (Simeone *et al*, 1987) and localization of *HOXC4* expression to the suprabasal layer of normal adult human skin (Reiger *et al*, 1994).

Taken together the two methods provide several important conclusions concerning expression of *HOX* genes during skin development. First, there appears to be a relatively conserved temporal and spatial expression pattern shared by many of the *HOX* genes. Expression appears to be limited to the basal cell layers in early skin development, prevalent in the basal and stratum granulosum layers of the epidermis as well as in the dermis during mid- to late second trimester development, and dramatically downregulated and restricted to the granular layers of the adult epidermis. No *HOX* gene expression was detected in adult dermis by either RT-PCR or *in situ* hybridization analysis among the subset of *HOX* genes analyzed. On the other hand, *HOX* genes appear to be expressed in unique patterns in the developing hair follicles. Secondly, the relative levels expression for the various *HOX* genes appeared to be constant throughout development. The *in situ* analysis showed, for instance, that *HOXA4* was always detected at a relatively high level in every tissue and time point analyzed, whereas *HOXB4* was only weakly expressed in each specimen examined. In addition, the PCR analysis showed the three *HOXA* genes to be the predominant genes detected at all time points studied.

It is unclear what the function(s) of the Hox proteins might be during skin development. In other developmental programs, Hox proteins appear to specify positional and timing information (Krumlauf, 1994). There have been several reports of differential *HOX* gene expression patterns that support this type of role for Hox proteins in skin and hair development (Chuong *et al*, 1990; Bieberich *et al*, 1991; Kanzler *et al*, 1994); however, there is increasing evidence that enforced expression of these genes in other tissues can lead to unregulated proliferation (Aberdam *et al*, 1991; Maulbecker and Gruss, 1993; Thorsteinsdottir *et al*, 1997), whereas loss of *HOX* gene expression is associated with increased apoptosis.[2] In the context of skin development, HOX proteins might function as gene activators to upregulate cellular proliferation in the early developing epidermis. Similar proliferative

[2]Izon DJ, Komuves LG, Rozenfeld S, Fong S, Largman C, Lawrence HJ: Loss of function of the homeobox gene HoxA-9 retards T-cell development and induces cell death. Manuscript submitted.

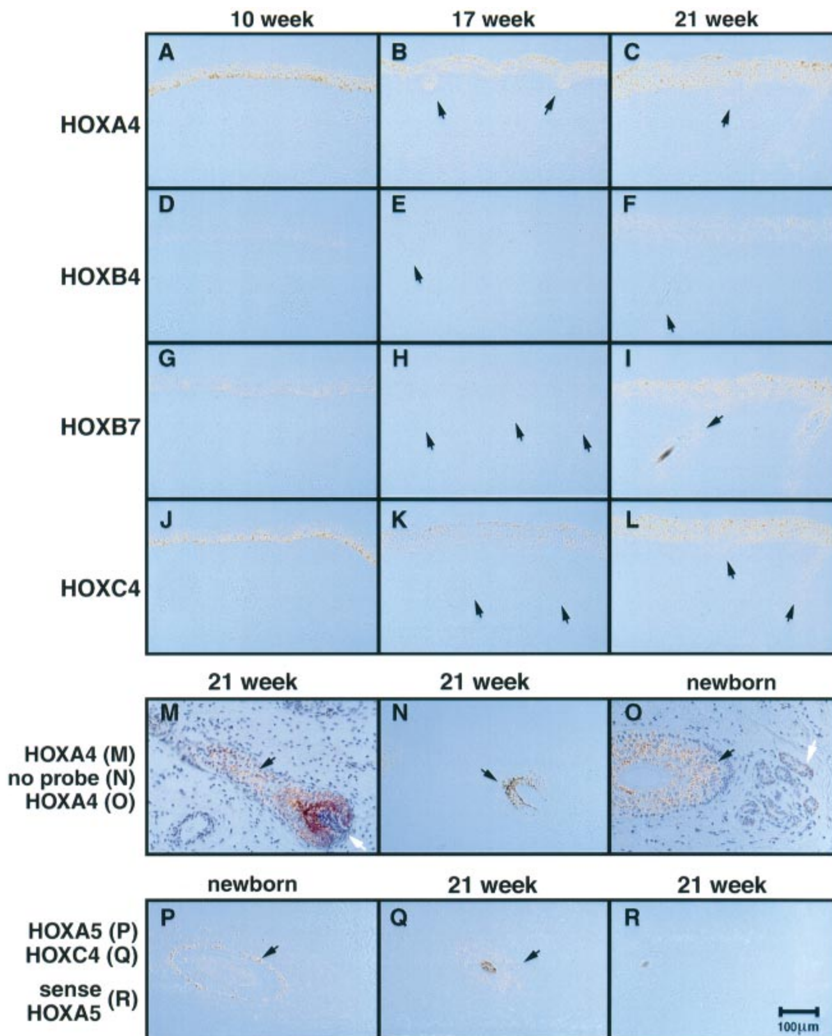


Figure 3. Expression of *HOXB4*, *HOXB7*, and *HOXC4* in developing skin. *In situ* hybridization analysis was used to localize *HOX* homeobox gene expression during embryonic skin development. For parts (*A*)–(*L*), expressions in 10 wk (*left panels*), 17 wk (*middle panels*), and 21 wk (*right panels*) scalp skin are shown. *HOX* genes studied were: *HOXA4* for comparison and for visualization in the absence of hematoxylin counter-staining (*A–C*); *HOXB4* (*D–F*); *HOXB7* (*G–I*); and *HOXC4* (*J–L*). For parts (*B*)–(*L*), the → point to developing hair follicles. Expression of *HOX* genes in hair follicles is also shown in (*M*)–(*Q*), as follows: *HOXA4* expression (*black* →) in a hair follicle from 21 wk skin (*M*), the white → denotes the lack of expression in the dermal papilla; the parallel section hybridized with a sense control probe to show the background due to the developing hair shaft (→) (*N*); *HOXA4* expression in a hair follicle (*black* →) and sweat glands (*white* →) from newborn skin (*O*); *HOXA5* expression in the inner root sheath (→) of the hair follicle from newborn skin (*P*); HOXC*4* expression in the developing hair follicle (→) in 21 wk skin (*Q*). Part (*R*) was hybridized with a control sense *HOXA5* RNA probe. Sections (*A*)–(*L*) were adjacent to those stained with hematoxylin to visualize skin development shown in **Fig 2**(***A***,***C***,***D***), respectively. Parts (*M*) and (*O*) were counter-stained with hematoxylin to visualize morphologic structures. Expression of these genes was visualized identically using a standard 5 min color development. *Scale bar*, 100 µm.

functions might also be associated with the increased activity associated with the dermis during second trimester skin development. In this regard, it should be noted that first and early second trimester skin possesses the capacity to heal wounds without scar formation, whereas third trimester and adult skin lack this attribute (Weeks and Nath, 1993; Nath *et al*, 1995). It is possible that HOX proteins within the dermis provide information for proper regeneration of the wound during embryonic development. Finally, we hypothesize that HOX proteins might function as repressors to downregulate cellular proliferation in the outer layers of the mature skin. Although few biologic targets of HOX protein regulation have been identified, several of the suggested targets of homeodomain protein action are cellular adhesion or signaling molecules, which could play a role(s) in cellular proliferation (Jones *et al*, 1992; White *et al*, 1992; Tomotsune *et al*, 1993).

The hypothetical roles described above would require HOX proteins to act as activators in one cellular environment and as repressors in a different cellular layer. One mechanism by which the HOX transcription factors might exhibit different phenotypic effects is through the presence or absence of partner proteins. We and others have recently shown that HOX proteins form cooperative DNA binding complexes with members of the PBX family of non-HOX homeodomain proteins (Chang *et al*, 1995, 1996; Lu *et al*, 1995; Phelan *et al*, 1995; van Djik *et al*, 1995). Furthermore, a recent model for HOX protein function proposes that they cooperatively bind DNA as activators in the presence of PBX and function as transcriptional repressors by binding alone to DNA (Pinsonneault *et al*, 1997). Thus one model would be that the HOX proteins might function as activators in early skin development by binding DNA with PBX or other protein partners, and might function as repressors in the granular cells of the adult epidermis if the array of partner proteins was different in these cells.

It is somewhat puzzling that there appears to be such a high redundancy of *HOX* gene expression in the developing skin. While *Hox* genes are expressed in developing murine spinal cord or limb buds in redundant, overlapping patterns, there are also apparent areas in which individual *Hox* genes show increased relative expression (Krumlauf, 1994). This does not appear to be the case in the developing skin, because the expression patterns for the six *HOX* genes examined appeared very similar. In addition, the expression of each gene appeared to be relatively uniform across the body. Although the level of our analysis was insufficient to determine if multiple *HOX* genes were expressed in individual cells, it appears likely that there is a high level of redundancy at the cellular level. Although this complexity is difficult to interpret in terms of functional roles for the homeoproteins, it should be noted that signaling molecules such as BMP-2 and BMP-4 (Francis *et al*, 1994), or the Wnt-like proteins (Parr and McMahon, 1994) that are thought to be both regulators and perhaps targets of HOX proteins (Iler *et al*, 1995; Manak *et al*, 1995), are themselves expressed in complex patterns throughout vertebrate development. In addition, functional *Hox* gene redundancy in murine rhombomere formation has recently been demonstrated (Gould *et al*, 1997). While the purpose of this redundancy is not clear, we have recently demonstrated large differences in complex stabilities between various HOX proteins bound to DNA with PBX (Shen *et al*, 1997). We hypothesize that the protein products derived from the HOX genes, which we have demonstrated to be expressed in developing skin, function by competing for DNA binding sites both as complexes with a variable

set of partner proteins and as independent DNA binding proteins. As a first step in defining the regulatory targets for HOX proteins in the skin, it will be necessary to elucidate the set of DNA binding partners expressed throughout skin development.

This work was supported by a grant from the American Plastic Surgery Education Foundation/Ethicon Research Fellowship to Eric Stelnicki, by the Research Service of the Department of Veterans Affairs (C.L.), NIH Grant #HD-25505 (S.A.), and by the G. Harold and Leila Y. Mathers Charitable Foundation (M.H.). We wish to thank members of the Largman laboratory for assistance and helpful comments. and Dr. Peter Elias for reading the manuscript. We also wish to thank the following individuals for supplying human HOX cDNA clones: Dr. Judy Gasson (HOXA5), Dr. Debbie Wolgemuth (HOXA4), and Dr. Eduardo Boncinelli (HOXA7, HOXB4, and HOXC4). A partial genomic clone containing the 3′ nonhomeobox coding and untranslated regions of human HOXA7 was the kind gift of Dr. Guy Sauvageau.

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