Vitamin D receptor-mediated control of Soggy, Wise, and Hairless gene expression in keratinocytes

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Abstract

The vitamin D receptor (VDR), but not its hormonal ligand, 1,25-dihydroxyvitamin D3 (1,25D), is required for the progression of the mammalian hair cycle. We studied three genes relevant to hair cycle signaling, DKKL1 (Soggy), SOSTDC1 (Wise), and HR (Hairless), to determine whether their expression is regulated by VDR and/or its 1,25D ligand. DKKL1 mRNA was repressed 49–72% by 1,25D in primary human and CCD-1106 KERTr keratinocytes; a functional vitamin D responsive element (VDRE) was identified at −9590 bp in murine Soggy. Similarly, SOSTDC1 mRNA was repressed 41–59% by 1,25D in KERTr and primary human keratinocytes; a functional VDRE was located at −6215 bp in human Wise.

In contrast, HR mRNA was upregulated 1.56- to 2.77-fold by 1,25D in primary human and KERTr keratinocytes; a VDRE (TGGTGAgtgAGGACA) consisting of an imperfect direct repeat separated by three nucleotides (DR3) was identified at −7269 bp in the human Hairless gene that mediated dramatic induction, even in the absence of 1,25D ligand. In parallel, a DR4 thyroid hormone responsive element, TGGTGAggccAGGACA, was identified at +1304 bp in the human HR gene that conferred tri-iodothyronine (T3)-independent transcriptional activation. Because the thyroid hormone receptor controls HR expression in the CNS, whereas VDR functions in concert with the HR corepressor specifically in skin, a model is proposed wherein unliganded VDR upregulates the expression of HR, the gene product of which acts as a downstream comodulator to feedback-repress DKKL1 and SOSTDC1, resulting in integration of bone morphogenic protein and Wnt signaling to drive the mammalian hair cycle and/or influencing epidermal function.

Key Words
- gene regulation
- vitamin D
- transcription factors
- hormone receptors
- skin

Introduction

Molecular control of the mammalian hair cycle is incompletely characterized. Three gene products that are involved in this process are hairless (HR), the vitamin D receptor (VDR), and retinoid X receptor-α (RXRα). Loss-of-function mutations in any of the genes encoding these proteins in mammals result in failure to reactivate the hair cycle after the loss of the first coat of hair, leading to alopecia and dermal cysts (Bergman et al. 2005). It has therefore been proposed that these three gene products function together in a single pathway to initiate a new hair cycle (Wang et al. 2007, Hsieh et al. 2010). However, further details about this pathway and the other gene products
that might be included have not been elucidated, although there is evidence that multiple signaling pathways are involved, including Wnt proteins (Fuchs et al. 2001), sonic hedgehog (Teichert et al. 2010), and bone morphogenetic proteins (BMPs; O’Shaughnessy et al. 2004).

Several clues have emerged from mouse gene knockout experiments, including a hairless gene ablation study by Thompson and colleagues (Beaudoin et al. 2005) that showed an inverse relationship between the expression of hairless and sclerostin domain-containing protein 1 (SOSTDC1) genes, the latter also known as Wnt modulator in surface ectoderm (Wise). SOSTDC1-encoded Wise inhibits both the Wnt and BMP signaling pathways in the course of the hair cycle (Lintern et al. 2009). Thompson and colleagues proposed that Hr suppression of Sostdc1 expression is important in triggering reinitiation of the anagen phase of the hair cycle by allowing keratinocytes to respond to an undetermined signal that presumably impinges on the hair-cycle-controlling pathways, including the Wnt-β-catenin pathway (Beaudoin et al. 2005).

Another gene with a possible role in the hair cycle is dickkopf-like 1 (DkkL1), encoding soggy. It has been reported that mRNA levels of both DkkL1 and Sostdc1 are upregulated in hr null mice, downregulated in hr-overexpressing transgenic mice, and repressed in keratinocytes that have been ‘rescued’ by expression of the hr gene under the control of a keratin-14 promoter (Zarach et al. 2004, Thompson et al. 2006). Dkk1 is a member of the Dickkopf family of secreted proteins, several of which regulate signaling by the canonical Wnt pathway (Niehrs 2006). Thompson et al. (2006) have shown that DkkL1 expression is measurable in hair follicles of mice and peaks during late anagen and early catagen. In contrast, the mammalian Hr protein, which has been shown to be essential for progression of the hair cycle, exhibits a complementary expression pattern, i.e. strong expression during early anagen, with expression declining sharply at the anagen–catagen transition (Panteleyev et al. 2000). These authors put forth a model wherein the Hr protein suppresses the expression of DkkL1 (Thompson et al. 2006) and also a second protein, Wise (Beaudoin et al. 2005). The suppression of both proteins is proposed to play a permissive role in allowing Wnt signaling to initiate a new anagen phase of the hair cycle.

Mammalian Hr is a highly conserved 130 kDa transcription factor that, according to in situ hybridization analysis in mice (Cachon-Gonzalez et al. 1999), is chiefly expressed in skin, cartilage, retina, inner ear, brain, colon, and oral/tongue/urogenital epithelia. Loss-of-function mutations in the human HR gene cause atrichia with papular lesions (Ahmad et al. 1999, Klein et al. 2002) and mutations leading to overexpression of HR protein also result in a hair–skin phenotype known as Marie Unna hereditary hypotrichosis (Wen et al. 2009, Ramot et al. 2010). In the hr/hr mouse (Cachon-Gonzalez et al. 1994), a mutation leading to partial loss of Hr protein causes disappearance of most hair after completion of the first hair cycle, along with dermal cysts, whereas a total loss of HR function, such as from a premature stop codon (Cachon-Gonzalez et al. 1999), results in complete alopecia after 3–4 weeks, but also thickened and wrinkled skin with many dermal cysts. Hr and VDR have been shown to physically and functionally interact (Hsieh et al. 2003a). VDR activates transcription in response to 1,25-dihydroxy-vitamin D3 (1,25D) by forming a heterodimer with one of the RXRs and binding to a vitamin D responsive element (VDRE) in or near each target gene (Whitfield et al. 2005). One consequence of a VDR/Hr interaction is that Hr inhibits the ability of VDR to activate transcription of its target genes in response to the 1,25D ligand (Hsieh et al. 2003a, Xie et al. 2006). Hr has also been shown to attenuate transactivation by the thyroid hormone receptor (TR; Potter et al. 2001), as well as the retinoic acid receptor-related orphan receptor-α (RORα; Moraitis & Giguere 2003).

The molecular basis of Hr-mediated transrepression of VDR, TR, and RORα signaling is not well characterized. It has been proposed that Hr exerts its repression via its Jumonji C-like domain to recruit histone deacetylases, which modify chromatin structure to silence gene transcription (Wang et al. 2007, Hsieh et al. 2010). It has also been reported that HR possesses intrinsic histone 3 lysine 9 demethylase activity, possibly controlling transcription catalytically via the histone code as an epigenetic ‘eraser’ (Liu et al. 2011). An interesting feature of human HR, as opposed to homologs reported from other mammalian species, is the presence of two isoforms, generated via alternative mRNA splicing. The α isoform (HRα) is produced from a complete mRNA transcript, whereas the β isoform (HRβ) is translated from a transcript that lacks exon 17 (Malloy et al. 2009).

Based upon its functioning as a corepressor of VDR, we tested the hypothesis that HR may target VDR–VDRE signaling and subsequently modulate downstream DKKL1 and SOSTDC1 expression. In silico analysis of the regions surrounding the DkkL1 and SOSTDC1 genes revealed candidate VDREs, two of which are shown herein to be functionally active. We further hypothesized that HR may be transcriptionally activated by VDR, reasoning that the resulting HR corepressor could reciprocally suppress HR expression and VDR mRNA, thus establishing a novel
inhibitory feedback loop to control the level of both proteins. In this study, an in silico search for potential hormone responsive elements in human HR led to the identification of an apparent constitutive VDRE 5' of the promoter, as well as a novel, ligand-independent thyroid hormone responsive element (TRE) in the first intron.

Materials and methods

Cell culture

Cell lines were cultured in the following media: human CCD-1106 KERTr (KERTr) in keratinocyte serum-free medium (Invitrogen) with the recommended additives; human Caco-2 colorectal adenocarcinoma and green monkey COS7 kidney, human HEK-293 embryonic kidney, and human HaCaT keratinocyte cells were all cultured in DMEM supplemented with 10% fetal bovine serum (FBS), with the latter two lines receiving supplements of 4 and 2 mM L-glutamine respectively. Normal primary neonatal human epidermal keratinocytes (HEKn) were purchased from Invitrogen and were cultured in serum-free EpiLife medium containing the recommended HKGS supplement kit reagents. Primary keratinocytes were incubated for 24 h in 1.2 mM calcium to elicit differentiation, which was evidenced by a clear change in morphology, with the uniform monolayer at 60 μM calcium transforming to dense/compacted, differentiated keratinocytes. Thus, for the present experiments, KERTr and HaCaT keratinocytes were undifferentiated, whereas HEkN cells were used in the differentiated state.

Plasmid constructs

Oligonucleotides corresponding to four copies of candidate VDREs along with four bases of flanking sequence on either side were separately annealed and inserted into the HindIII and BglII sites of pLuc-MCS (Stratagene Corp., La Jolla, CA, USA). Positive control reporter plasmids were similarly constructed with four copies of the rat osteocalcin VDRE (rOC-Luc) or 5500 bp from the promoter of the human CYP24A1 gene (p24OHaseLuc). The integrity of each of these reporter vectors was confirmed by DNA sequencing. The expression vector for WT rat Hr (pRK5myc-rhr), which contains the CMV promoter, has been described previously (Potter et al. 2001). An expression vector for full-length human HR (HRα) was kindly provided by Dr. A Hillem (Rheinsche Friedrich-Wilhelms-Universitat, Germany). HRα is cloned into the mammalian expression vector p3xFLAG-CMV-7.1 with an expression driven by the CMV promoter, yielding HRα with a triple FLAG-tag at the N-terminus. An expression vector for HRβ (HRa1072-1126) was derived from the parent HRα vector using the Quickchange XL Mutagenesis Kit (Stratagene). The expression plasmids for human VDR (pSG5hVDR) and for human TRβ1 have been described previously (Hsieh et al. 1991). The reporter construct used for assaying TR signaling contained two copies of the TRE from the rat myosin heavy chain gene (Hsieh et al. 1991).

Transcriptional activity assays

Cultured cells were plated in 24-well plates at a density of 80,000 cells/well in 1 ml of the appropriate medium. Six or more hours following plating (when the cells had become attached), wells were cotransfected using Lipofectamine and PLUS Transfection Reagents (Invitrogen) with 250 ng/well of reporter plasmid (either the rOC-Luc reporter plasmid or one of the VDRE-Luc or TRE-Luc constructs), along with pRL-CMV (1 ng/well), pSG5hVDR (250 ng/well), and 499 ng of pHZ18U plasmid as carrier DNA. For each well, plasmids (total 1.0 μg) were diluted into 25 μl serum-free medium. PLUS reagent (4 μl) was added and incubated at room temperature for 15 min. Lipofectamine Reagent (2 μl) was diluted into 25 μl serum-free medium in a second tube. The pre-complex DNA and Lipofectamine tubes were combined and incubated for an additional 15 min. The final DNA–Plus–Lipofectamine complexes (54 μl/well) were added to the cultured cells and incubated for 48 h with or without 1,25D. The wells were washed twice with PBS and lysed with 150 μl passive lysis buffer (from DLR Assay Kit, Promega). Firefly and Renilla luciferase activities were measured sequentially from each lysate using a Sirius Luminometer (Titertek Berthold, Pforzheim, Germany) and a Dual Luciferase Assay Kit (Promega) as per the manufacturer’s instructions. The ratio of firefly:Renilla luciferase activity was calculated to normalize for transfection efficiency.

In silico, bioinformatic search for candidate VDREs

The search criteria were based on the following direct repeat 3 (DR3) degenerate sequence, with hexanucleotide repeats in upper case and the three nucleotide spacer in lower case: RGKDBRnnrRGKDBR, where R=A or G, K=G or T, D=G, T or A, B=G, T or C, and n=any base.

Electrophoretic mobility shift assay

Annealed, 32P-labeled oligonucleotides were used in an electrophoretic mobility shift assay (EMSA) as follows.
Double-stranded oligonucleotides encoding two tandem copies of the candidate VDREs plus four bases of flanking sequences and four-base overhangs were labeled with $^{32}$PdCTP and used along with *Escherichia coli*-expressed, partially purified human VDR and RXRx as described previously (Hsieh et al. 2003b). A double-stranded oligonucleotide, containing a dual copy of the VDRE sequence from the rat osteocalcin gene, served as a positive control that binds VDR and RXR. Reaction mixtures were resolved on 4% nondenaturing polyacrylamide gels, dried, and exposed to Amersham Hyperfilm at room temperature.

**Quantitative real-time PCR assays**

KERTr and HEKn were plated at 800 000 cells/100 mm plate in 10 ml of DMEM with 10% FBS. The cells were treated with $10^{-8}$ M 1,25D for 24 h and harvested by trypsinization. Total RNA was extracted using a High Pure RNA isolation kit (Roche Applied Science) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1.0 µg total RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories). The cDNA was used in 20 µl PCRs containing 10 µl FastStart Universal SYBR Green Master Mix (Roche Applied Science) with primers. Reactions were performed on an ABI 7500 Fast instrument (Life Technologies). Data were analyzed using the comparative Ct method as a means of relative quantitation, normalized to an endogenous reference (glyceraldehyde phosphate dehydrogenase (GAPDH)) and relative to a calibrator (normalized Ct value from vehicle-treated cells), and expressed as $2^{-\Delta\Delta Ct}$ according to Applied Biosystems’ User Bulletin 2: Rev B, ‘Relative Quantitation of Gene Expression’. The primer sets for real-time PCR were as follows: Human HR primers were 5'-GGGACACATCGATAGGAACAAGGAT-3' (forward primer) and 5'-TATGTCCTGAAGTCCCGGGTCC-3' (reverse primer). Primers for human GAPDH expression were 5'-ACACTTTGTATGAGAACAGGAC-3' (forward primer) and 5'-CAGGGATGATGTTCCTGGAGAGC-3' (reverse primer). Primers for human DKKL1 were 5'-GACAGACGAGGGAGGTG-3' (forward primer) and 5'-TCAAATCACCCTGCAAGGTCC-3' (reverse primer). Primers for human SOSTDC1 were 5'-TGTTCCATACTTGGAGAT-3' (forward primer) and 5'-AAGCCGTCCTGCAACCAATCACCTGAT-3' (reverse primer). Human CYP24A1 was detected using forward primer 5'-CAGGGATGATGTTCCTGGAGAGC-3' and reverse primer 5'-TCTTCTTCTCACAACAGGACAG-3'.

**Results**

The 1,25D hormone regulates the expression of genes relevant to skin/hair cycle

Assuming Hr functions as a corepressor in cooperation with VDR to modulate genes encoding factors that ensure hair cycle progression, we initially examined several genes observed to be overexpressed in keratinocytes of *hr*-ablated mice. As illustrated in Fig. 1, we evaluated the effect of $10^{-8}$ M 1,25D for 24 h on the expression of *DKKL1*, *SOSTDC1*, and *HR* in cultured human keratinocytes using qRT-PCR. *CYP24A1* was employed as a highly induced positive control for VDR-mediated 1,25D action to ensure that the tested cells expressed VDR and thereby responded to the 1,25D ligand in the expected fashion. Indeed, Fig. 1A illustrates the dramatic 89- and 124-fold enhancement of *CYP24A1* mRNA concentrations elicited by the vitamin D hormone in undifferentiated KERTr and differentiated primary human keratinocytes, respectively, indicating that both systems express approximately equal VDR levels and represent valid models to probe VDR-targeted gene expression. Furthermore, we observed that CASP14, which is a nonapoptotic caspase family

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**Figure 1**

1,25D effect on *CYP24A1, DKKL1, SOSTDC1, and HR* expression in keratinocytes. A (*CYP24A1*), B (*DKKL1*), C (*SOSTDC1*), D (*HR*), Real-time PCR was performed on total RNA prepared from: KERTr (center black bars in each panel) and primary normal human epidermal keratinocytes (HEKn; right black bars in each panel) using specific primers for each gene, as described in Materials and methods. All results were normalized to GAPDH, and cells treated with $10^{-8}$ M 1,25D for 24 h were compared with vehicle ethanol (EtOH) control, which was set to 1.0-fold. Each bar represents the average of three independent experiments $\pm$ s.d. *All 1,25D treated groups were statistically significantly different from EtOH control (*P*<0.05), with the exception of *HR* in HEKn cells (*P*<0.08), although this latter induction by 1,25D has been observed in three additional HEKn primary cultures to average 1.40-fold (data not shown).
member that is essential for keratinocyte differentiation (Rendl et al. 2002), is induced 2.11-fold by 1,25D in KERTr cells (data not shown), a finding verified by cDNA microarray analysis (Haussler et al. 2013). Thus, despite their immortality through transformation by human papillomavirus 16 E6/E7, KERTr cells apparently retain the cadre of comodulators required for 1,25D/VDR influence on skin/hair cycle relevant genes.

In accordance with this, the data in Fig. 1B reveal that 1,25D represses DKKL1 mRNA concentrations by 72 and 49% in KERTr and normal primary human keratinocytes respectively. Thus, DKKL1 fits the concept of repression by the VDR–RXR complex in cooperation with Hr, with relieving of this repression (Beaudoin et al. 2005) in the keratinocytes from hr-null mice. As depicted in Fig. 1C, 1,25D also represses SOSTDC1 mRNA levels by 41 and 59% in KERTr and primary human keratinocytes respectively. Therefore, like DKKL1, SOSTDC1 fits the concept of repression by the VDR–RXR complex in cooperation with Hr, with relieving of this repression (Beaudoin et al. 2005) in the keratinocytes from hr-null mice. With respect to regulation of human HR expression by 1,25D, qRT-PCR results illustrated in Fig. 1D reveal a 2.77- and 1.56-fold enhancement of HR mRNA expression by 1,25D treatment of KERTr and primary human keratinocytes respectively. Thus, we hypothesized that VDR binds to VDREs in the HR gene to activate transcription, and in DKKL1 and SOSTDC1 to repress transcription.

**Regulation of DKKL1 and SOSTDC1 by 1,25D**

We first investigated the mechanism whereby 1,25D controls DKKL1 and SOSTDC1, noting that VDREs have yet to be identified in the vicinity of either gene. Consequently, in silico analysis was performed on both mouse and human DKKL1 genes in order to locate potential VDREs. As shown in Fig. 2A for mouse DkkL1, a single candidate VDRE, GGGTCAtgAGGGCA, was located at −9590 bp relative to the transcriptional start site. A single candidate VDRE was also found in the human DKKL1 gene at −9830 bp (Fig. 2B). Both candidates were analyzed via an EMSA to test their in vitro VDR-binding capabilities. As shown in Fig. 2C, only the mouse candidate VDRE was capable of binding VDR–RXR complex when compared with the rOC VDRE positive control. Transcriptional activity of the mouse VDRE was examined using the dual luciferase assay procedure. As shown in Fig. 2D, the −9590 VDRE is activated by 1,25D, driving liganded, VDR-mediated transcriptional activation with a 12-fold effect over the vehicle control, approaching the activity of the human CYP24A1 natural promoter fragment (5.5 kb) containing a classic dual VDRE (Fig. 2D), and exceeding the activity of the rat osteocalcin VDRE (Fig. 2E). As shown in Fig. 2E, activation of the −9590 VDRE by 1,25D is reduced from 16.7- to 2.5-fold by rat Hr, similar to VDREs in well-characterized, 1,25D-regulated genes. Curiously, the basal activity of the −9590 VDRE is increased by approximately a factor of two in the presence of the rHr, a phenomenon not usually observed for VDREs, wherein both basal and 1,25D-stimulated transcriptions are suppressed. The mechanistic caveats regarding the murine −9590 VDRE in DkkL1 are that it is: i) specific to the mouse, as a conserved homolog has yet to be located in the human DKKL1 gene and ii) functioning as a positive VDRE out of the keratinocyte setting, whereas 1,25D represses DKKL1 expression in intact keratinocytes (Fig. 1B).

Similar to DKKL1, SOSTDC1 expression is repressed by 1,25D in keratinocytes. (Fig. 1C), and both genes are overexpressed in keratinocytes derived from hr knockout mice. Given these observations, it is evident that both genes are regulated by 1,25D/VDR and by Hr, probably involving a pattern of comodulators specific to differentiated keratinocytes. Further experiments probing the human SOSTDC1 gene were conducted via in silico analysis, yielding the identification of two potential human SOSTDC1 VDREs (Fig. 3A). An EMSA revealed that both the −6215 and −5857 bp responsive elements in human SOSTDC1 were capable of binding a VDR–RXR complex, in vitro (Fig. 3B). However, as shown in Fig. 3C, only the −6215 element was capable of mediating repression of transcription by 1,25D even though, in the same experiment, 1,25D dramatically induced transcription directed by the mouse DkkL1 VDRE located at −9590 as well as by the human CYP24A1 promoter fragment. As depicted in Fig. 3D, in a repeat experiment, the −6215 bp VDRE mediated significant repression of reporter transcription in the presence of 1,25D, a phenomenon that is best shown in the left panel of Fig. 3D. This repression is reproducible in the presence of endogenous levels of VDR in HEK-293 cells (bars in right panel of Fig. 3D, ‘no added VDR’). Thus, the data in Fig. 3 indicate that the molecular mechanism whereby 1,25D represses SOSTDC1 expression in human cells probably involves a negative VDRE at −6215 bp in the SOSTDC1 gene.

**Identification and functional analysis of a putative VDRE in the human HR gene**

In silico searches revealed six candidate VDREs, located at −7269, −7410, −8171, −9087, −23 029, and
J-C HSIEH and others

VDR controls Hairless, Wise, and Soggy in skin

Figure 2

In vitro characterization and functional analysis of an in silico-identified VDRE in the mouse DkkL1 gene. (A and B) Bioinformatic identification of candidate VDREs in the vicinity of the mouse and human DKKL1 genes respectively. (C) EMSA of VDR/RXR binding to candidate VDREs as described in Materials and methods. Lane 1 contains the rat osteocalcin (rOC) VDRE probe plus added RXRα (50 ng) and VDR (100 ng); the shifted complex is indicated by an arrow at the left. Lanes 2 and 5 contain labeled probes with no protein added to the candidate mouse and human VDREs respectively. Lanes 3 and 6 contain probes plus added (50 ng) RXRα only. Lanes 4 and 7 each contain respective probes plus added RXRα (50 ng) and VDR (100 ng). (D) Reporter gene assays testing the functionality of the mouse DKKL1 candidate VDRE in COS7 cells. A double-stranded oligonucleotide corresponding to the −9590 VDRE was inserted as a four copy tandem repeat upstream of a luciferase reporter gene and cotransfected into COS7 cells with a pSG5VDR expression vector to supply exogenous VDR. The human CYP24A1 5.5 kb natural promoter-luciferase construct was used as a positive control. Indicated wells received 1,25D (10⁻⁸ M final concentration) at 18 h post-transfection. After an additional 24 h, cell lysates were harvested and luciferase activity was assayed as described in Materials and methods. Each bar represents the average of three independent experiments performed in triplicate ± s.d. *1,25D-treated groups statistically significantly different from the ETOH control (P < 0.05). (E) Rat Hr inhibits transactivation by VDR in the −9590 DkkL1 VDRE-luciferase construct. COS7 cells were cotransfected with the following plasmids as indicated: pSG5hVDR (250 ng/well), pRK5rHr (200 ng/well), and the −9590 DkkL1 VDRE luciferase reporter (250 ng/well), or a positive control plasmid containing four copies of the rat osteocalcin VDRE. Transcriptional activities were quantified by luciferase assay and all values were normalized to the expression of Renilla luciferase. Each bar represents the average of three independent experiments performed in triplicate ± s.d. *1,25D-treated groups statistically significantly different from the ETOH control (P < 0.05).

−24 780 bp relative to the transcriptional start site of the HR gene (Ahmad et al. 1999; Fig. 4A, upper). It was next determined whether any of these candidate VDREs could bind VDR, in vitro, via EMSA. As shown in Fig. 4A (lower), VDR-RXRα complexes were formed in the absence of 1,25D ligand on four of the six tested VDREs: the −7269 element (lane 12), the −7410 element (lane 10), the −8171 element (lane 8), and a weak complex on the −24 780 element (lane 2). Luciferase assays revealed that only the −7269 VDRE is capable of conferring 1,25D induction onto the reporter gene in HEK-293 cells (Fig. 4B). It is noteworthy that transcription of the −7269 VDRE-linked reporter gene is significantly upregulated even in the absence of 1,25D, although the addition of ligand results in a further increase by 3.5-fold (Fig. 4B). The −7269 VDRE performs similarly in HaCaT keratinocytes (Fig. 4C), with significant ligand-independent activity and approximately a twofold augmentation of transcription by 1,25D. In COS7, however, the VDRE is completely ligand-independent in the mediation of transactivation (Fig. 4D). These data indicate that VDR-mediated transactivation driven by the HR −7269 VDRE is cell-type-specific in terms of degree of ligand autonomy. An unusual feature of this VDRE is the presence of a T nucleotide at the first (underlined) position of the VDRE (TGGTGAAGTGGTCA). We suggest that the presence of a thymidine in this position might confer ligand-independent regulation onto a reporter gene.
Functional interaction of −7269 VDRE with human HR isoforms and rat Hr protein

To examine whether transactivation mediated by the −7269 VDRE is repressed by Hr, and whether this repression affects 1,25D-dependent transcription, ligand-independent transcription, or both, HEK-293 cells were cotransfected with hVDR and Hr expression plasmids along with the −7269 VDRE-reporter construct, and 1,25D-induced transcriptional activity was measured in the absence and presence of 10−8 M of 1,25D (Fig. 4E). Cotransfection of either HRα or HRβ resulted in a dramatic repression of ligand-independent VDRE–VDRE transcription (~70%) and a milder reduction in transcriptional activation by 1,25D-ligated VDR–VDRE (~30%), with no significant differences between the two human HR isoforms. In contrast, rat Hr sharply repressed both ligand-independent and 1,25D-dependent trans-activation, almost completely blunting any effect of 1,25D (Fig. 4E, right two bars). Although rat Hr resides in the pRK5-myc vector, whereas human HR isoforms lie in the p3xFLAG-CMV-7.1 vector, both vectors employ the strong CMV promoter, yielding efficient expression. Western blotting (data not shown) analysis confirmed that human HRα and HRβ are equally expressed, but lack
of an effective antibody prevented the quantitation of rat Hr expression. Nevertheless, because rat Hr proved to be more potent than the human isoforms in suppressing VDR-mediated transactivation, there is little doubt about the activity of rat Hr as a VDR corepressor.

**Characterization of a novel TRE in the human Hr gene**

A 3 kb portion of the human Hr promoter (−2902 to +102) was previously shown to be differentially regulated by T₄ in neuroblastoma cells and keratinocytes, and a TRE (AGGGCAAttgAGGACA) was localized to −2632 to −2647 bp upstream of the human Hr gene (Engelhard & Christiano 2004). Because functional VDREs apparently accommodate a thymidine in the first position (such as the −7269 element), the search for TRE sequences was expanded to include such half-sites to determine if a novel TRE might be found in the human Hr gene. A candidate TRE with the sequence TGGTGAggccAGGACA was indeed identified at +1304 to +1319 in the

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**Figure 4**

*In vitro* characterization and functional analysis of the *in silico*-identified VDREs in the human Hr gene. (A, upper panel) Bioinformatic identification of candidate VDREs in the vicinity of the human Hr gene. Locations and sequences of candidate VDREs relative to the Hr transcriptional start site (Hr start) are depicted employing an image from the UC Santa Cruz web browser (hg18 version; Kent et al. 2002). As in Fig. 3 (legend), CTCF binding sites were used as boundaries for the genomic interval to be searched. Orientation of each candidate element is indicated as sense (S) or antisense (AS). Neighboring genes include receptor accessory protein 4 (REEP4). (A, lower panel) Annealed, 32P-labeled oligonucleotides were used in an EMSA as described in Materials and methods and the legend to Fig. 2. Each bar represents the average of three independent experiments performed in triplicate ± S.D. *+1,25D-treated groups statistically significantly different from ETOH control (P<0.05). (C and D) The −7269 VDRE-luciferase construct was tested in HaCaT (C) and COS7 (D) cell lines. Each bar represents the average of three independent experiments performed in triplicate ± S.D. *+1,25D-treated groups statistically significantly different from ETOH control (P<0.05). (E) The ability of human Hr isoforms to inhibit transactivation by VDR. HEK-293 cells were cotransfected with the following plasmids as indicated: pShuttleVDR (250 ng/well), p3xflagCMVHRα (200 ng/well), p3xflagCMVHRβ (200 ng/well), pRK5HRα (200 ng/well) and the −7269 VDRE luciferase reporter (250 ng/well). Transcriptional activities were quantified by luciferase assay and all values were normalized to the expression of Renilla luciferase. Each bar represents the average of three independent experiments performed in triplicate ± S.D. *+1,25D-treated groups statistically significantly different from ETOH control (P<0.05). hairless-treated groups statistically significantly different from untreated control (P<0.05).
first intron of the human HR gene (Fig. 5A). Evaluation of the transcriptional enhancer capacity of the −2632 and +1304 TREs revealed that transcription of the −2632 TRE-linked reporter is activated only in the presence of T3 (10−7 and 10−8 M), with very modest ligand-independent activity (Fig. 5B). In contrast, the +1304 TRE conferred significant luciferase expression in the absence of T3 that was not further enhanced by the addition of T3 (Fig. 5C), unveiling a ligand-independent TRE analogous to the constitutive −7269 VDRE in the HR gene (Figs 4D and 5D).

**Figure 5**

TREs in the human HR locus. (A) A previously reported TRE at −2632 along with a novel TRE at +1304 bp in the context of the human HR gene. (B and C) Reporter gene assays of each TRE in the presence of either TRβ alone or TRβ + T3 (two concentrations as indicated) for 24 h. Mock transfected wells that received empty reporter plasmid are shown for comparison. Each bar is the compilation of three independent experiments performed in triplicate ± s.e. *T3-treated groups statistically significantly different from EtOH control ( P < 0.05). Ligand independence is demonstrated in (C) by the fact that −T3 and + T3 values are not statistically different ( P > 0.1). (D) Sequence comparison of the novel ligand-independent TRE and VDRE sequences, the thymidine residue at the first (n1) position of each responsive element are highlighted with a box. Half-element sequences are identical between the VDRE and TRE, with differences only in the three and four nucleotide spacers.

**Discussion**

Control of the hair cycle by VDR is crucial in pathobiology because hair serves a critical function in terrestrial mammals by protecting skin from DNA-damaging u.v. irradiation. Rachitic, VDR-null mice display the phenotype of alopecia and dermal cysts, which is not ameliorated by a high calcium, lactose, and phosphate rescue diet that reverses the calcium and bone mineral defects (Sakai et al. 2001). However, there exists no corresponding pathological phenotype in the skin of mice that unable to synthesize 1,25D, suggesting that at least a part of the action of VDR in skin is independent of the 1,25D ligand.

The hair and skin abnormalities observed in mice with hr loss-of-function mutations are largely recapitulated in VDR knockout mice (Miller et al. 2001), an observation that is consistent with a functional interaction between the VDR and Hr nuclear proteins in signal transduction pathways that drive the hair cycle. Because Hr is a nuclear receptor corepressor, it is presumably this action of Hr-VDR in mammals that is required for the progression of the hair cycle. Following this reasoning, Thompson and colleagues (Beaudoin et al. 2005) have defined Sostdc1, Dkk1, and Casp14 as genes overexpressed in keratinocytes from hr-null mice, and Kato and colleagues (Yamamoto et al. 2009) characterized S100A8 and CASP14 as two genes overexpressed in VDR-null keratinocytes. It was previously observed (Haussler et al. 2010) that S100A8 is rapidly repressed by 1,25D in human keratinocytes. Similar to S100A8, SOSTDC1 is significantly repressed by 1,25D-activated VDR in KERtr and primary human keratinocytes (Fig. 1C). Suppression of SOSTDC1 mRNA by 1,25D was verified using reverse transcriptase PCR in human keratinocytes (Haussler et al. 2010), and cDNA microarray analysis of Caco2 cells (data not shown). The present results demonstrate the interaction of VDR with a VDRE in the human SOSTDC1 gene at −6215 bp (Fig. 3B), and this VDRE elicited 1,25D-dependent repression when linked in a reporter construct, indicating that it acts as a negative VDRE (Fig. 6, upper right). Because SOSTDC1-encoded Wise not only antagonizes the Wnt pathway by binding to lipoprotein-receptor-related protein (LRP) but also inhibits the BMP pathway through neutralization of BMP4 (Lintern et al. 2009), repression of SOSTDC1 by VDR-Hr could constitute a major event in initiation of the mammalian hair cycle (Fig. 6). However, the relationship between SOSTDC1 repression (as well as HR induction) by 1,25D and progression of the hair cycle is unclear, because post-morphogenic responses to VDR in the mammalian hair follicle are vitamin D ligand-independent.
Figure 6
Model for VDR and Hr action in keratinocytes. Regulation of hr, Sostdc1, and DkkL1 expression by 1,25D/VDR may contribute to epidermal cell functions as depicted in the central and right portions of the model. However, along with VDR (Cianferotti et al. 2007), and Hr (Cachon-Gonzalez et al. 1999), β-catenin is absolutely required in keratinocytes (Huelsken et al. 2001) to permit mammalian hair cycling, and the ligand-independent action of VDR to drive the hair cycle is thought to involve Wnt signaling in skin stem cells (Beaudoin et al. 2005, Cianferotti et al. 2007). Thus, as illustrated in the left portion of the figure, by upregulating HR, and in turn repressing Sostdc1 (Wise) and DkkL1 (Soggy) expression, unliganded VDR could conceivably drive the hair cycle. Signaling in the mammalian hair cycling is complex, consisting of the convergence of two signaling pathways, BMP and Wnt. Noggin from the dermal papilla initially antagonizes BMP4 signaling in bulb (or bulge) keratinocytes, allowing for signaling pathways, BMP and Wnt. Noggin from the dermal papilla initially antagonizes BMP4 signaling in bulb (or bulge) keratinocytes, allowing for the accumulation of Lef1/TCF, a transcriptional coactivator that targets antagonizes BMP4 signaling in bulb (or bulge) keratinocytes, allowing for signaling pathways, BMP and Wnt. Noggin from the dermal papilla initially antagonizes BMP4 signaling in bulb (or bulge) keratinocytes, allowing for the accumulation of Lef1/TCF, a transcriptional coactivator that targets genes via DNA-binding partners such as β-catenin. Cessation of Noggin signaling reinstates BMP signal transduction via SMADs provided that Wise (encoded by Sostdc1), which antagonizes both Wnt and BMP pathways (Lintern et al. 2009), has also been repressed, either directly by VDR (upper right), indirectly through VDR induction of Hr (right center), or by a combination thereof. Wnt ligand (e.g. Wnt 10b) signaling leads to accumulation of β-catenin, which cooperates with Lef1/TCF to induce genes encoding factors such as sonic hedgehog (Shh), that trigger the hair cycle to transition from telogen (resting) to anagen (growth). Finally, the figure is not meant to imply that keratinocytes and hair follicle bulge stem cells are the same population of cells. Because mature keratinocytes rather than hair follicle stem cells that express Sox9, K15, Cd34, and alpha 6, were used for the present experiments, one must be cautious in applying this model to control of the hair cycle over regulation of more straightforward mature epidermal cell functions. See text for additional discussion. Abbreviations not defined in the text are: HDMe, histone demethylase; Wnt, ortholog of Drosophila wingless and mouse int-1; Lef1, lymphoid enhancer factor-1; TCF, T cell-specific factor; msx-1 and msx-2, orthologs of Drosophila muscle-specific homeobox protein. Factors that are membrane receptors or transporters are boxed. Solid arrows indicate activation and dotted lines ending in a continuous perpendicular line denote inhibition.

As illustrated in Fig. 1B, DKKLI mRNA also is repressed by 1,25D in KERTr and primary human keratinocytes. DkkL1 is expressed in the hair follicle in a manner inversely related to the expression of Hr (Thompson et al. 2006), leading to the suggestion that DkkL1 may be a Wnt inhibitor. Should this be the case, DkkL1 suppression by VDR-Hr would potentiate the action of the Wnt signaling pathway to initiate a new cycle of hair growth. Indeed, Wnt is the major inductive signaling pathway activated in hair follicle stem cells (HFSCs) during the onset of a new hair cycle, and the VDR does play a role in HFSC function, although in an apparently unliganded fashion, and perhaps in cooperation with Hr to repress DkkL1 expression. One cautionary note this hypothesis is that DkkL1 expression does not affect Wnt signaling in the testes (Kohn et al. 2005). However, it is possible that DkkL1 lacks the activity to inhibit Wnt signaling in the testes but possesses such activity in the context of the hair cycle. Alternatively, other major Wnt regulators such as wifi1, Dkk2, msx, etc., may instead replace DkkL1 as a pivotal Wnt regulator modulated by VDR-Hr. A second cautionary note is that the data in Fig. 2D are seemingly in conflict with those in Fig. 1B showing repression, because 1,25D is observed to upregulate transcription driven by a murine DkkL1-linked reporter gene in Fig. 2D. Thus, the murine 9590 DkkL1 VDRE performs like a classic ‘positive’ VDRE in isolation (Fig. 2C and D), but considering that 1,25D represses DKKLI expression in intact keratinocytes (Fig. 1B), this VDRE apparently adopts a repressive character in its natural context of DNA and associated co-modulators in the differentiated keratinocyte. We term such VDREs ‘conditional’, rather than attempting to classify them as ‘positive’ or ‘negative’. Indeed, the direction and/or magnitude of DKKLI gene control may...
be cell-context specific as well as differentiation stage selective, with repression mediated by the VDR–RXR–HR–HDAC complex bound to the VDRE (corepressors shown at lower right; Fig. 6; Skorija et al. 2005). In isolation, outside the context of the differentiated keratinocyte, this repression of DKKL1 is postulated to be relieved by VDR liganding with 1,25D in conjunction with recruitment of coactivators to the VDRE, effectively replacing HR/corepressors as depicted in Fig. 6 (lower right object). Although it is clear that DKKL1 expression is regulated by VDR, the mechanism via the murine −9590 Dkk1 VDRE appears to be species-specific, as we have yet to identify an active VDRE in the human DKKL1 gene to account for the repression by 1,25D shown in Fig. 1B. Moreover, we have on rare occasions observed in some preparations of calcium-differentiated primary human keratinocytes, but never in KERTr cells, that 1,25D induces rather than represses both DKKL1 and SOSTDC1 expression (data not shown), suggesting that both the DKKL1 and SOSTDC1 VDREs should perhaps be referred to as ‘conditional’. This observation, probably accounted for by incomplete differentiation of occasional primary human keratinocyte lots, is also consistent with the recent finding that HR elicits coactivation of the cathelicidin gene while corepressing CYP24A1 mRNA in the context of HaCaT keratinocytes (Chuma et al. 2012). Finally, although no dramatic epidermal or hair cycle phenotype exists in either Dkk1- or Sostdc1-knockout mice, the two gene products could redundantly regulate the hair cycle, requiring a double knockout to generate a hair cycle phenotype, or both gene products may coordinate with other genes within the skin compartment to control hair growth.

A major goal of the present experiments was to characterize the regulation and functions of the Hr comodulator. It has been demonstrated previously that Hr functions as a VDR corepressor (Hsieh et al. 2003a), raising the question as to whether Hr and VDR might reciprocally control one another via a counterregulatory feedback loop. Indeed as shown herein, activated VDR is a bona fide positive regulator of Hr mRNA expression (Fig. 1D). It has been reported that 816 bp of 5′ flanking sequence derived from the human Hr gene could support VDR-dependent transpression that was amplified by 1,25D whereas, paradoxically, the ‘full promoter’ supported ligand-independent transactivation by VDR in a cell-specific fashion (Engelhard et al. 2008), thus suggesting the existence of both negative and positive feedback loops whereby VDR modulates Hr expression. In this study, we report that human Hr gene expression is augmented by 1,25D in both keratinocyte (Fig. 1D) and enterocyte (Caco-2, data not shown) cell lines. It is therefore concluded that the induction of Hr by 1,25D-ligated VDR represents a feedforward counter-regulatory action to curtail the effects of the 1,25D hormone–VDR complex through the synthesis of its corepressor, HR.

In a quest to seek functional VDREs in the vicinity of the human Hr gene, we identified, in silico, the existence of a novel VDRE that is proposed to account for at least part of the ability of VDR to regulate Hr gene expression. This candidate VDRE, located 7269 bp 5′ of the transcription start site in the human Hr gene, confers varying degrees of ligand-dependent transactivation in HEK-293 (Fig. 4B), HaCaT (Fig. 4C), and COS7 (Fig. 4D) cells. The ability of the −7269 VDRE to activate luciferase also contains a significant ligand-independent component in HaCaT cells (Fig. 4C) and especially in COS7 cells (Fig. 4D). The molecular basis of this cell-type-specific activity is not defined as yet, but it may be generated by differential expression of transcriptional coactivators which are then recruited when RXR–VDR is conformed by docking on this particular responsive element, which we have designated as a ‘constitutive’ VDRE (Fig. 6, central right object). The phenomenon of vitamin D ligand-independent transactivation mediated by a VDRE has been reported by MacDonald and colleagues (Ellison et al. 2007) for the human CYP24A1 promoter, but only when it is transfected into normal human keratinocytes, and not into either transformed keratinocytes or normal fibroblasts. A foundation for ligand independence may reside primarily in the precise sequence of the VDRE, providing there exists support for VDR–RXR heterodimerization other than via ligand binding, as there apparently does in the case of the CYP24A1 promoter in normal human keratinocytes (Ellison et al. 2007). The conclusion herein is that vitamin D ligand-unoccupied VDR appears to be capable of transactivation of Hr via its constitutive VDRE (Fig. 6, centre right), triggering the switch for progression of the hair cycle through subsequent repression of SOSTDC1 (Fig. 6, upper right object) and possibly of DKKL1 (Fig. 6, lower right object). Alternative mechanisms explaining vitamin D ligand-independent transactivation by VDR include: VDR activation via phosphorylation by a cell-selective kinase, cell- or promoter-specific coactivation of unoccupied VDR–RXR, and endogenous synthesis of an alternative lipophilic, nonvitamin D VDR ligand.

The role of thyroid hormone and its receptor, TRβ, in the hair follicle (Billoni et al. 2000) does not appear to be as crucial as the roles of VDR and Hr. However, human patients who are hypothyroid or hyperthyroid (van Beek...
et al. 2008) or who have mutations in TRβ (Guran et al. 2009) show a diffuse hair loss or hair thinning, although neither perturbations in thyroid hormone status nor receptor ablation fully mimic the hr knockout phenotype (van Beek et al. 2008). In this study, we identified an atypical TRE, TGGTGAggccAGGACA, at +1304 bp within the first intron of the human HR gene (Fig. 5A). This novel TRE confers ligand-independent transactivation onto a heterologous reporter gene in COS7 cells (Fig. 5C). This study thus reveals the existence of two ligand-independent elements, a TRE and a VDRE, both of which contain a thymidine residue in the first position of the first half-element (Fig. 5D). A thymidine in this position is neither found in previously characterized DR3 VDREs (Whitfield et al. 2008) nor is it observed in consensus DR4 TREs (Umesono et al. 1991). We propose that a thymidine in this position, corresponding to the binding site for the RXR heterodimeric partner of either VDR (Jin & Pike 1996) or TR (Perlmann et al. 1993), may cause a conformational change in RXR that is then transferred to the primary receptor (VDR or TR), resulting in the ability of the heterodimer to attract coactivators and thereby function predominantly as a ligand-independent transactivator of the HR gene. The plausibility of such a mechanism was demonstrated in a recent study of VDR–RXR binding to two different VDRE sequences (Zhang et al. 2011), in which the point was made that VDR–RXR heterodimer binding to differing DNA sequences can relay information to the ligand-binding domain and specifically to the AF-2 C-terminal helix domain of VDR that makes contact with coactivators. It is therefore implied that unique nuclear receptor responsive elements may exist in genes which are regulated by unliganded nuclear receptor complexes, many of which are indeed observed bound to DNA in the human genome (Meyer et al. 2012). In conclusion, although the three novel VDREs identified herein exist in genes that are well established to affect the hair cycle, further research including chromatin immunoprecipitation-sequencing (ChIP-seq) experiments is required to prove that the present findings on soggy, Wise, and hairless close the gap in our biological understanding of control of the hair cycle and/or epidermal keratinocyte function.

Importantly, although the present communication emphasizes the findings as they may relate to the mammalian hair cycle, no actual hair cycle analyses were carried out. The current experiments used keratinocytes, not purified bulge stem cells, and no investigation of the functioning of bulge stem cells or their progeny after 1,25D treatment, in vivo, has been performed.

The observed regulation by 1,25D of HR, SOSTDC1, and DKKL1 could constitute epidermal keratinocyte phenomena. Thus, an alternative interpretation of the results herein is that they are more pertinent to epidermal keratinocyte function, than to regulation of the hair cycle, per se. Indeed, 1,25D induces the expression of a number of genes in cultured keratinocytes, the products of which are potential prodifferentiative and structural components, as well as detoxification, immunomodulation, and anti-inflammatory/anti-oxidation principles (Bikle 2012). For example, 1,25D induces caspase-14 in keratinocytes (Haussler et al. 2013). This nonapoptotic caspase is crucial for keratinocyte differentiation (Rendl et al. 2002). 1,25D induces cathelicidin and several defensins in keratinocytes (Bikle 2012), indicating that vitamin D modulates the immune complement in skin. Also, late cornified envelope (LCE-1D, -1F, -2B) genes in the epidermal differentiation complex are induced by 1,25D in human keratinocytes (Haussler et al. 2013). Finally, 1,25D increases the expression of a number of keratin-related gene products (Haussler et al. 2013) which, when considered along with the LCE proteins, indicates that vitamin D signaling supports the skin structurally and mediates barrier function development. Therefore, inductive epidermal and mesenchyme signaling after 1,25D treatment is more likely to influence specific epidermal cell properties than to play a part in the hair cycle as shown by previous studies (e.g. hair reconstitution assays).

In summary, VDR functions to drive the mammalian hair cycle in cooperation with Hr, primarily via the repression of gene expression, whereas 1,25D acts via VDR binding to signal transcription of genes for which the products support the development and barrier function of the skin.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This study was supported by National Institutes of Health grant NIH DK033351 to M R H, NIH CA140285 to P W J, and a grant from the Arizona Biomedical Research Commission to J-C H.

Author contribution statement
Acknowledgements
The authors are grateful to Dr Yifei Wu for technical assistance.

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Received in final form 30 October 2013
Accepted 4 November 2013
Accepted Preprint published online 4 November 2013