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

Jui-Cheng Hsieh[1], **Rudolf C Estess**[1], **Ichiro Kaneko**[1,2], **G. Kerr Whitfield**[1], **Peter W. Jurutka**[1,2], and **Mark R. Haussler**[1,*]

[1]Department of Basic Medical Sciences, University of Arizona College of Medicine, Phoenix, AZ 85004, USA

[2]School of Mathematical and Natural Sciences, Arizona State University, Phoenix, AZ 85306, USA

Abstract

The vitamin D receptor (VDR), but not its hormonal ligand, 1,25-dihydroxyvitamin D_3 (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 if 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 1.56–2.77-fold by 1,25D in primary human and KERTr keratinocytes; a VDRE (TGGTGAgtgAGGACA) consisting of an imperfect direct repeat separated by 3 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 T_3-independent transcriptional activation. Because thyroid hormone receptor controls *HR* expression in the central nervous system, 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 BMP and Wnt signaling to drive the mammalian hair cycle and/or influencing epidermal function.

Keywords

Gene regulation; Hormone receptors; Skin; Transcription factors; Vitamin D

[*]Corresponding author: Department of Basic Medical Sciences, University of Arizona College of Medicine-Phoenix, 425 North 5th Street, Phoenix, AZ 85004-2157, USA, haussler@email.arizona.edu (M.R. Haussler).

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.

AUTHOR CONTRIBUTIONS

Conception and design: Jui-Cheng Hsieh, G. Kerr Whitfield, Peter W. Jurutka, and Mark R. Haussler
Development of methodology: Jui-Cheng Hsieh, Rudolph Estess, G. Kerr Whitfield, and Ichiro Kaneko
Acquisition of data: Jui-Cheng Hsieh, Rudolph Estess, G. Kerr Whitfield, and Ichiro Kaneko
Writing, review, and/or revision of the manuscript: Jui-Cheng Hsieh, G. Kerr Whitfield, Peter W. Jurutka, and Mark R. Haussler
Administrative, technical, or material support: Jui-Cheng Hsieh, G. Kerr Whitfield, Peter W. Jurutka, and Mark R. Haussler
Study supervision: Jui-Cheng Hsieh and Mark R. Haussler

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 re-initiate 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 (Hsieh, *et al.* 2010; Wang, *et al.* 2007). 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 morphogenic 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 re-initiation 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^{-} 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 (Thompson, *et al.* 2006; Zarach, *et al.* 2004). DkkL1 is a member of the Dickkopf family of secreted proteins, several of which regulate signaling by the canonical Wnt pathway (Niehrs 2006). Thompson and colleagues (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 hairless protein (Hr), 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/nasal/bladder/urethral 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 (Ramot, *et al.* 2010; Wen, *et al.* 2009). 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-dihydroxyvitamin D_3 (1,25D) by forming a heterodimer with one of the retinoid X receptors (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 also has 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 (Hsieh *et al.* 2010; Wang *et al.* 2007). 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 the present 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 Corp., Carlsbad, CA) with the recommended additives; human Caco-2 colorectal adenocarcinoma and green monkey COS-7 kidney, human HEK-293 embryonic kidney and human HaCaT keratinocyte cells were all cultured in DMEM supplemented with 10% FBS, with the latter two lines receiving supplements of 4 mM and 2 mM L-glutamine, respectively. Normal primary neonatal human epidermal keratinocytes (HEKn) were purchased from Invitrogen Corp., Carlsbad, CA 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 utilized 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 Cholla CA). 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 wild-type rat Hr (pRK5myc-rhr), which contains the CMV promoter, was described previously (Potter *et al.* 2001). An expression vector for full length human HR (HRα) was kindly provided by Dr. A. Hillmer (Rheinische Friedrich-Wilhelms-Universität, Germany). *HRα* is cloned into the mammalian expression vector p3xFLAG-CMV-7.1 with expression driven by the CMV promoter, yielding HRα with a triple FLAG-tag at the N-terminus. An expression vector for HRβ (HRΔ1072–1126) was derived from the parent HRα vector utilizing the Quickchange XL Mutagenesis Kit (Stratagene, La Jolla, CA). The expression plasmids for human VDR (pSG5hVDR) and for human TRβ1 have been described previously (Hsieh, *et al.* 1991). The reporter construct utilized 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 per well in 1 mL of the appropriate medium. Six or more hours following plating (when the cells become attached), wells were cotransfected using Lipofectamine and PLUS transfection reagents (Invitrogen Corp., Carlsbad CA) 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 pTZ18U 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 minutes. 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 minutes. The final DNA-Plus-Lipofectamine complexes (54 μl per well) were added to the cultured cells and incubated for 48 hrs with or without 1,25D. Wells were washed twice with PBS and lysed with 150 μl passive lysis buffer (from DLR assay kit, Promega, Madison, WI). Firefly and Renilla Luciferase activities were measured sequentially from each lysate using a Sirius Luminometer (Pforzheim, Germany) and a Dual Luciferase assay kit (Promega Corp.) as per the manufacturer's instructions. The ratio of firefly to 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 (EMSA)

Annealed, ^{32}P-labeled oligonucleotides were used in an electrophoretic mobility shift assay 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 [α-^{32}P]dCTP and utilized along with *E. coli*-expressed, partially purified human VDR and RXRα 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 per 100 mm plate in 10 ml of DMEM with 10% fetal bovine serum. The cells were treated with 10^{-8} M 1,25D for 24 hr and harvested by trypsinization. Total RNA was extracted using a High Pure RNA isolation kit (Roche Applied Science, Indianapolis IN) 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, Hercules CA). 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, Carlsbad, CA). Data were analyzed using the comparative Ct method as a means of relative quantitation, normalized to an endogenous reference (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." Primer sets for real-time PCR were as follows. Human *HR* primers were 5′-GGGACACATCGATAGGGAACAAGGAT-3′ (forward primer) and 5′-TATGTCCTGAAGTCCCGGGTCC-3′ (reverse primer). Primers for human glyceraldehyde phosphate dehydrogenase (hGAPDH) expression were 5′-ACAACTTTGGTATCGTGGAAGGAC-3′ (forward primer) and 5′-CAGGGATGATGTTCTGGAGAGC-3′ (reverse primer). Primers for human *DKKL1* were 5′-GACAACAAGACAGGAGAGGTG-3′ (forward primer) and 5′-TCAAATCACCCTCGAAGCTC-3′ (reverse primer). Primers for human *SOSTDC1* were 5′-TGTTCCATAGCCTCCTCCAATCCAGTTA-3′ (forward primer) and 5′-AACTGCGTTCCACCAAATACATCTCTGAT-3′ (reverse primer). Human *CYP24A1* was detected using forward primer 5′-CAGCGAACTGAACAAATGGTCG-3′ and reverse primer 5′-TCTCTTCTCATACAACACGAGGCAG-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 Figure 1, we evaluated the effect of 10^{-8} M 1,25D for 24 hours 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 non-apoptotic caspase family 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.

Accordingly, 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 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 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, GGGTCAtggAGGGCA, 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 electrophoretic mobility shift assay (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 to the rOC VDRE positive control. Transcriptional activity of the mouse VDRE was examined using the dual luciferase assay procedure. As shown in Figure 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 (see Fig. 4D), wherein both basal and 1,25D-stimulated transcription are suppressed. The mechanistic caveats to the murine −9590 VDRE in *DkkL1* are that it is: a) specific to the mouse, as a conserved homolog has yet to be located in the human *DKKL1* gene, and b) 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, likely 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 both the −6215 bp and −5857 bp responsive elements in human SOSTDC1 were capable of binding a VDR-RXR complex, *in vitro* (Fig. 3B). However, as shown in Figure 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 viewed 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 likely 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 −23029 and −24780 bp relative to the transcriptional start site of the *HR* gene (Ahmad *et al.* 1999) (Fig. 4A, top). It was next determined whether any of these candidate VDREs could bind VDR, *in vitro*, via EMSA. As shown in Fig. 4A (bottom), 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 −24780 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 of 3.5-fold (Fig. 4B). The −7269 VDRE performs similarly in HaCaT keratinocytes (Fig. 4C), with significant ligand-independent activity and approximately a 2-fold augmentation of transcription by 1,25D. In COS-7, however, the VDRE is completely ligand-independent in mediating 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 (TGGTGAgtgAGGTCA). We hypothesize 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 hairless isoforms and rat hairless 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-VDR transcription (~70%) and a milder reduction in transcriptional activation by 1,25D-liganded 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 transactivation, 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) 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 as to 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 T_3 in neuroblastoma cells and keratinocytes, and a TRE (AGGGCAtctgAGGACA) was localized −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 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 T_3 (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 T_3 that was not further enhanced by the addition of T_3 (Fig. 5C), unveiling a ligand-independent TRE analogous to the constitutive −7269 VDRE in the *HR* gene (Fig. 4D; Fig. 5D).

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 UV irradiation. Rachitic, VDR-null mice display the phenotype of alopecia and dermal cysts, which is not ameliorated with 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 pathologic phenotype in the skin of mice 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 progression of the hair cycle. Following this reasoning, Thompson and coworkers (Beaudoin *et al.* 2005) have defined *SOSTDC1*, *DkkL1* 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 utilizing reverse transcriptase PCR in human keratinocytes (Haussler *et al.* 2010), and cDNA microarray analysis of Caco-2 cells (data not shown). The present results demonstrate VDR interaction 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 object). Because *SOSTDC1*-encoded Wise not only antagonizes the Wnt pathway by binding to 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 initiating 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.

As illustrated in Fig. 1B, *DKKL1* 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 caution to 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 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 wif1, DKK2, msx, etc., may instead replace *DKKL1* as a pivotal Wnt regulator modulated by VDR-Hr. A second caution 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 (Figs. 2C and 2D), but considering that 1,25D represses *DKKL1* expression in intact keratinocytes (Fig. 1B), this VDRE apparently adopts repressive character in its natural context of DNA and associated comodulators 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 *DKKL1* 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 in lower right object, Fig. 6) (Skorija, *et al.* 2005). In isolation, outside the context of the differentiated keratinocyte, this repression of *DKKL1* is postulated 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 *DkkL1* 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 observed in Fig. 1B. Moreover, we have on rare occasions observed in select 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 *DkkL1*- 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 possibly 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 vitamin D receptor 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 transrepression 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 the present 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-liganded VDR represents a feedforward counterregulatory 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 COS-7 (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 COS-7 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 comodulators 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, central right object), 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, non-vitamin 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 the present study, we identified an atypical TRE, TGGTGAggccAGGACA, at +1304 bp within the first intron of the h*HR* gene (Fig. 5A). This novel TRE confers ligand-independent transactivation onto a heterologous reporter gene in COS-7 cells (Fig. 5C). The present 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.* 2005) 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 as a predominantly 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 impact the hair cycle, further research including 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 utilized keratinocytes, not purified bulge stem cells, and no investigation of bulge stem cell or progeny function after 1,25D treatment, in vivo, has been performed. The observed regulation by 1,25D of *HR, SOSTDC1*, and *DkkL1* could conceivably 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 pro-differentiative 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 non-apoptotic 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.

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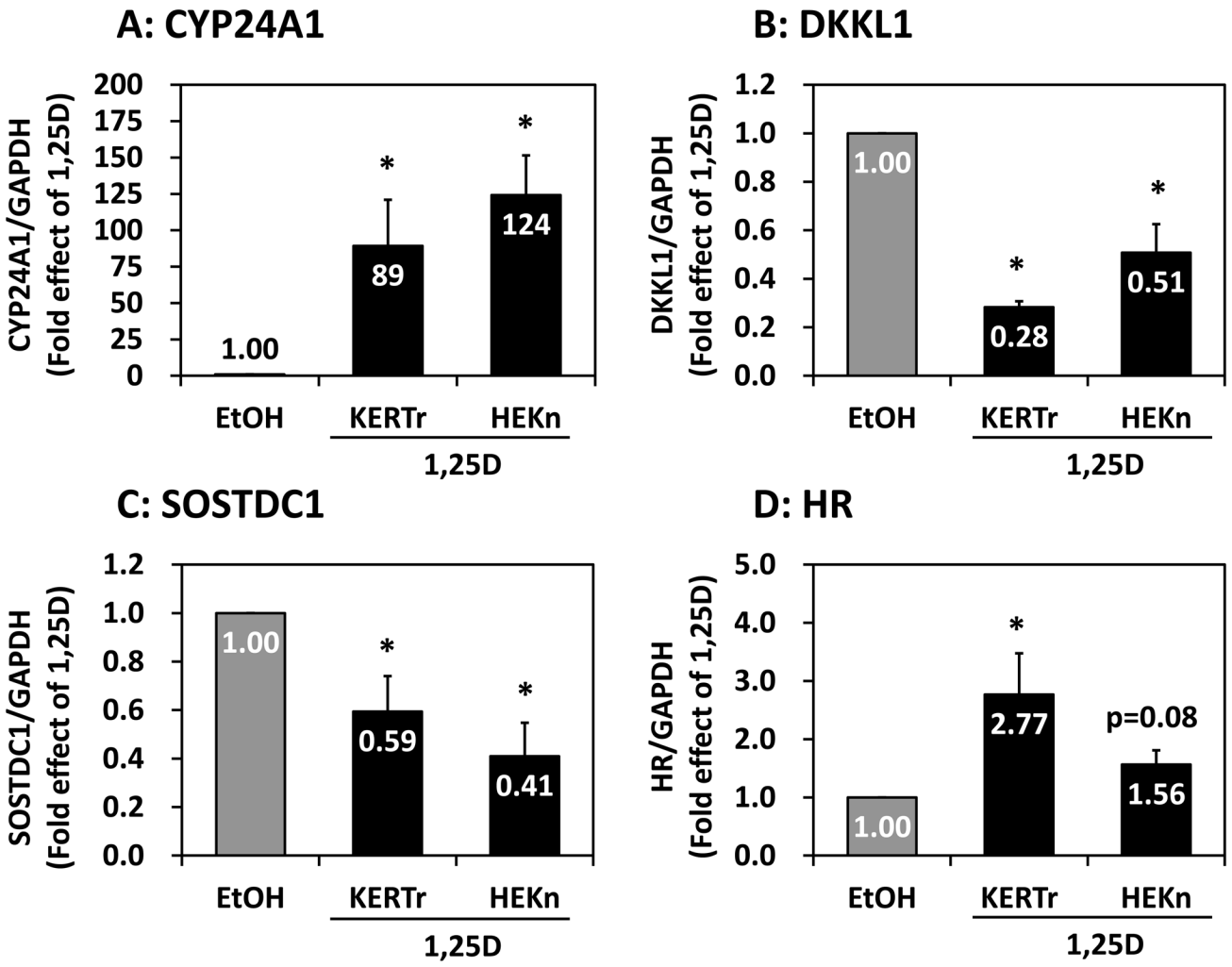

Figure 1. 1,25D effect on *CYP24A1*, *DKKL1*, *SOSTDC1*, and *HR* expression in keratinocytes

A *(CYP24A1)*, **B *(DKKL1)***, **C *(SOSTDC1)***, **D *(HR)***, Real time PCR was performed on total RNA prepared from: KERTr (center solid bars in each panel) and primary normal human epidermal keratinocytes (HEKn) (right solid bars in each panel) using specific primers for each gene, as described in Methods. All results were normalized to GAPDH, and cells treated with 10^{-8} M 1,25D for 24 hr were compared to vehicle (ETOH) control, which was set to 1.0-fold. Each bar represents the average of 3 independent experiments ± standard deviation. *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).

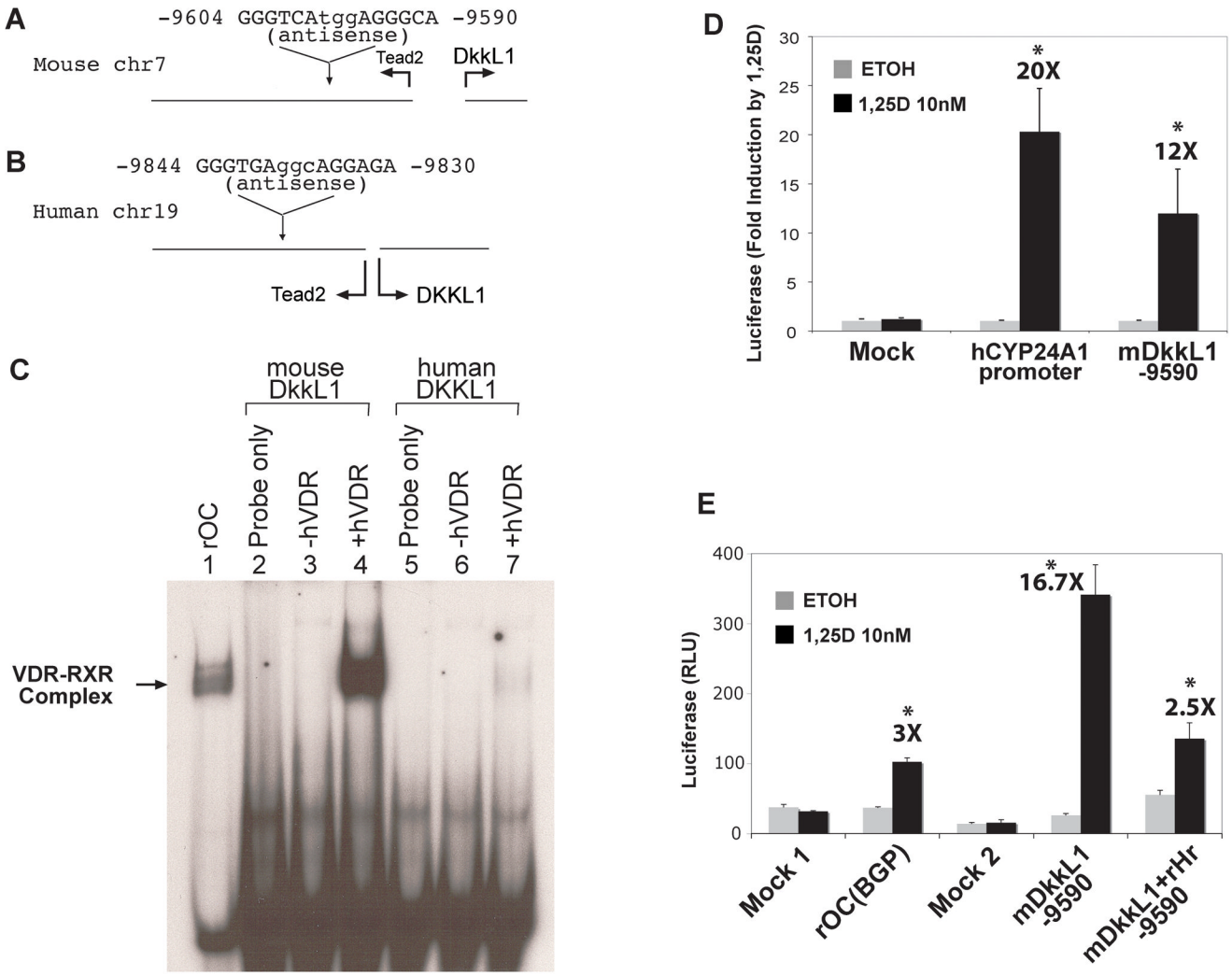

Figure 2. ***In vitro*** **characterization and functional analysis of an** ***in silico*****-identified VDRE in the mouse** ***DkkL1*** **gene**

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 Methods. Lane 1 contains the rat osteocalcin (rOC) VDRE probe plus added RXRα (50 ng) and VDR (100 ng); the shifted complex is indicated with an arrow on 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 COS-7 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 COS-7 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 hours post-transfection. After an additional 24 hours, cell lysates were harvested and luciferase activity was assayed as described in Methods. Each bar represents the average of three independent experiments performed in triplicate ± standard deviation. *1,25D-treated groups statistically significantly different from ETOH control ($p<0.05$). **E,** Rat Hr inhibits

transactivation by VDR in the −9590 *DkkL1* VDRE-luciferase construct. COS-7 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 ± standard deviation. *1,25D-treated groups statistically significantly different from ETOH control ($p<0.05$).

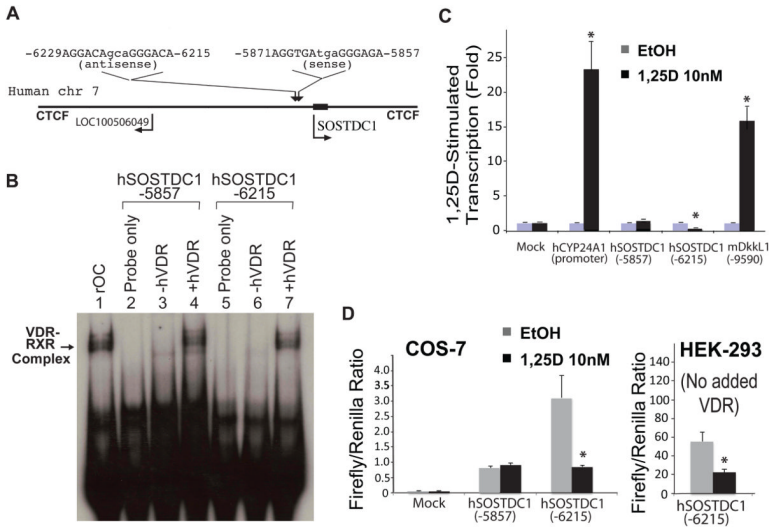

Figure 3. *In vitro* characterization and functional analysis of *in silico*-identified VDREs in the *SOSTDC1* gene

A, Bioinformatic identification of candidate VDREs in the vicinity of the human *SOSTDC1* gene. Binding sites for the CTCF insulator protein were determined in normal human epidermal keratinocytes (Mikkelsen, *et al.* 2007); these sites were used to delineate the genomic region that was searched. **B,** Annealed, ^{32}P-labeled oligonucleotides were used in EMSA as described in Methods and the legend to Fig. 2. **C,** Reporter gene assays testing the functionality of the human *SOSTDC1* candidate VDREs in COS-7 cells. Double-stranded oligonucleotides corresponding to the −5857 and −6215 VDREs were evaluated by luciferase assay as described in Methods and the legend to Fig. 2; data are expressed as fold-stimulation of transcription by 1,25D treatment on the ordinate. The luciferase constructs containing the human *CYP24A1* natural promoter and the −9590 mouse *DkkL1* VDRE were used as positive controls. Mock represents empty reporter vector transfections. Each bar represents the average of three independent experiments performed in triplicate ± standard deviation. *1,25D-treated groups statistically significantly different from ETOH control ($p<0.05$). **D,** Reporter gene assays testing the functionality of the human *SOSTDC1* candidate VDREs in COS-7 and HEK-293 cells. Experiments were carried out as described in C above, except that the data for the *CYP24A1* natural promoter positive control are not shown. *Left panel* represents a magnification of the data obtained in COS-7 in an independent experiment, excluding *CYP24A1*, allowing visualization of the repressive effect of the −6215 VDRE. *Right panel* illustrates an independent experiment in HEK-293 cells, wherein no exogenous VDR was included as indicated. Each bar represents the average of three independent experiments performed in triplicate ± standard deviation. *1,25D-treated groups statistically significantly different from ETOH control ($p<0.05$).

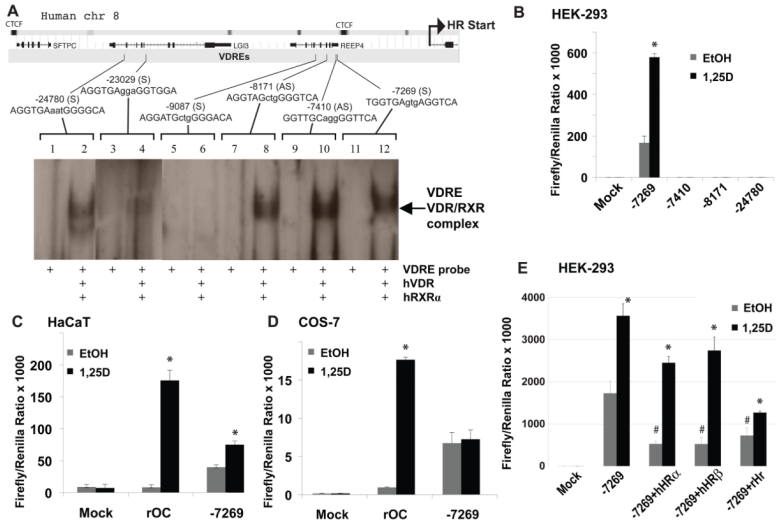

Figure 4. *In vitro* characterization and functional analysis of the *in silico*-identified VDREs in the human *HR* gene

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, ^{32}P-labeled oligonucleotides were used in an EMSA as described in Methods and the legend to Fig. 2. Even numbered lanes contained partially purified human VDR and RXRα proteins; odd numbered lanes contained probe only. **B,** Reporter gene assays testing the functionality of human *HR* candidate VDREs in HEK-293 cells. Double-stranded oligonucleotides corresponding to each *HR* VDRE that tested positively in the EMSA were separately evaluated by luciferase assay as described in Methods and in the legend to Fig. 2. Each bar represents the average of three independent experiments performed in triplicate ± standard deviation. *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 (panel C) and COS-7 (panel D) cell lines. Each bar represents the average of three independent experiments performed in triplicate ± standard deviation. *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: pSG5hVDR (250 ng/well), p3xflagCMVhHrα (200 ng/well), p3xflagCMVhHrβ (200 ng/well), pRK5rHr (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 ± standard deviation. *1,25D-treated groups statistically significantly different from ETOH control ($p<0.05$). #Hairless-treated groups statistically significantly different from untreated control ($p<0.05$).

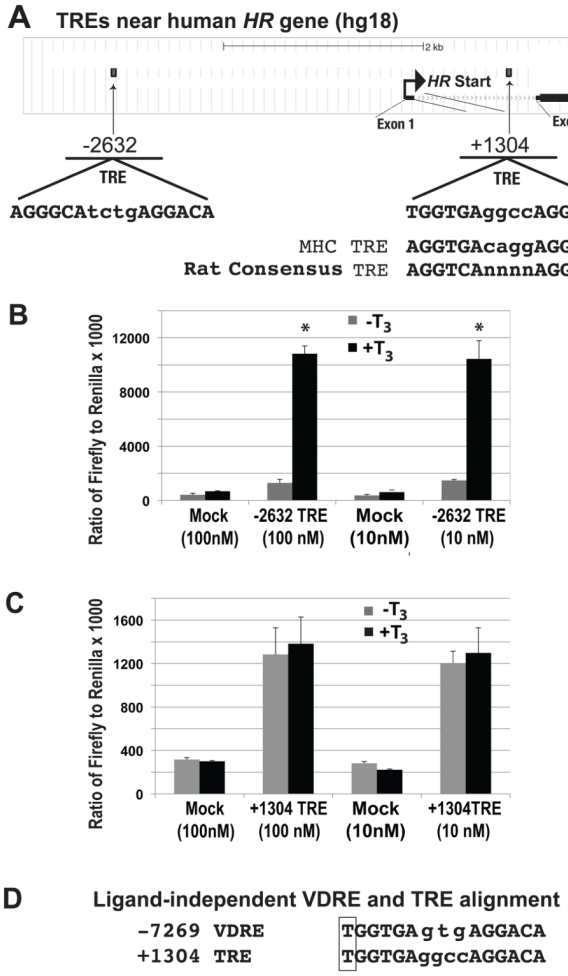

Figure 5. TREs in the human *HR* locus

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 + T_3 (two concentrations as indicated) for 24 hours. Mock transfected wells that received empty reporter plasmid are shown for comparison. Each bar is the compilation of three independent experiments performed in triplicate ± standard deviation. *T_3-treated groups statistically significantly different from ETOH control ($p<0.05$). Ligand-independency is demonstrated in **C** by the fact that $-T_3$ and $+T_3$ values are not statistically different ($P>0.1$). **D**, Sequence comparison of the novel ligand-independent TRE and VDRE sequences, boxing the thymidine residue at the first (n1) position of each responsive element. Half-element sequences are identical between the VDRE and TRE, with differences only in the 3 and 4 nucleotide spacers.

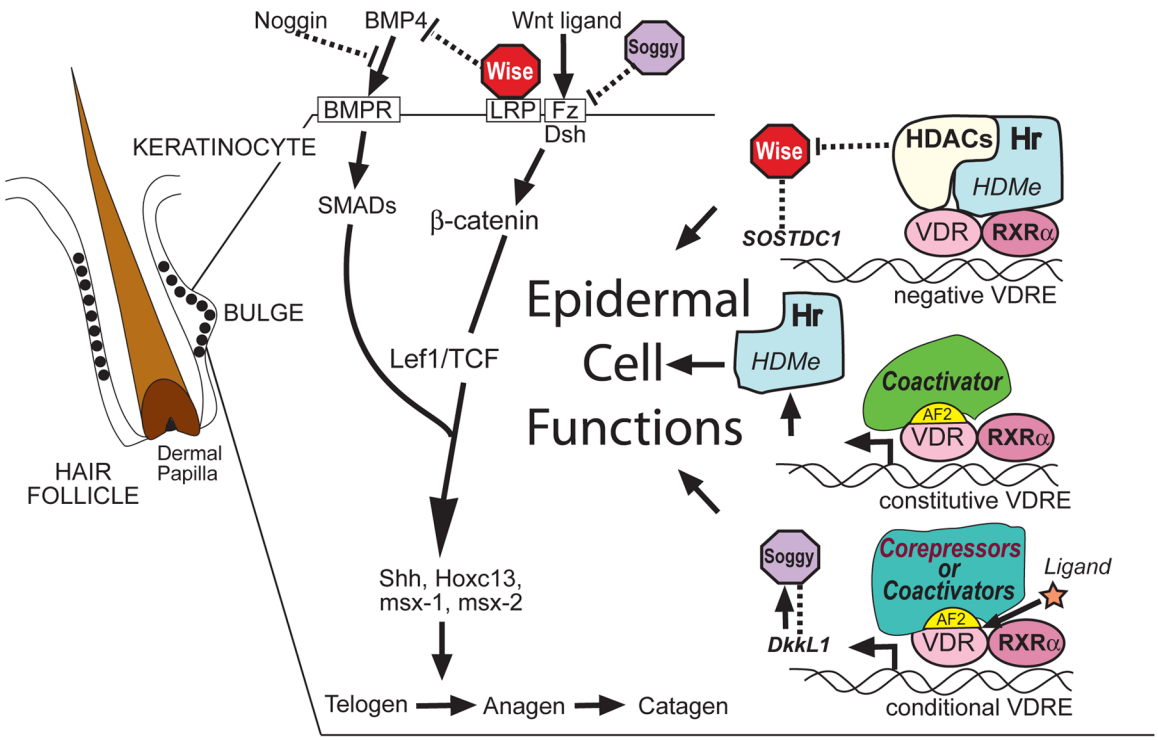

Figure 6. Model for VDR and Hr action in keratinocytes

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 model, 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 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 utilized 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 solid perpendicular line denote inhibition.