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Microarray Analysis Demonstrates a Role for Slug in Epidermal Homeostatsis

Kim M. Newkirk
University of Tennessee - Knoxville, knewkirk@utk.edu

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Kimberly M. Newkirk[1], Debra A. MacKenzie[2], Alan P. Bakaletz[3], Laurie G. Hudson[2] and Donna F. Kusewitt[1]

Slug (Snail2) is a member of the Snail family of zinc-finger transcription factors with regulatory functions in development, tissue morphogenesis, and tumor progression. Little is known about Slug in normal adult tissue; however, a role for Slug in the skin was suggested by our previous observations of Slug expression in normal murine keratinocytes and Slug induction at wound margins. To study the impact of Slug in the skin, we compared patterns of gene expression in epidermis from Slug-null and wild-type mice. A total of 139 genes had significantly increased, and 109 genes had significantly decreased expression in Slug knockout epidermis. Altered expression of selected genes in Slug knockout epidermis was validated by real-time PCR and immunohistochemistry. Previously reported Slug targets were identified, in addition to novel genes, including cytokeratins, adhesion molecules, and extracellular matrix components. Functional classification of altered gene expression was consistent with a role for Slug in keratinocyte development and differentiation, proliferation, apoptosis, adhesion, motility, as well as angiogenesis and response to environmental stimuli. These results highlight the utility of genetic models to study the *in vivo* impact of regulatory factors in unperturbed skin and suggest that Slug has significant activities in the adult epidermis.

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INTRODUCTION

The first member of the Snail family of transcription factors, Snail, was identified 20 years ago in *Drosophila*. Vertebrates have three Snail family genes: *Snail1* (*Snail*), *Snail2* (*Slug*), and *Snail3* (*Smuc*). The Snail family transcription factors are recognized regulators of epithelial-mesenchymal transformation (EMT) in development, and there is increasing evidence that these zinc-finger transcription factors also regulate EMT during tumor progression and metastasis (Nieto *et al.,* 1994; Savagner *et al.,* 1997; Sefton *et al.,* 1998; Hemavathy *et al.,* 2000; Savagner, 2001; Nieto, 2002; Shook and Keller, 2003; Barrallo-Gimeno and Nieto, 2005). It has also been suggested that Snail family members more broadly regulate cell movement and adhesion rather than EMT *per se* (Barrallo-Gimeno and Nieto, 2005). Snail is essential for early embryogenesis and Snail-null mutants die at gastrulation (Sefton *et al.,* 1998). In contrast, Slug-null mice are viable and reproduce, despite some abnormalities including small body size, reduced fertility, minor craniofacial defects, pigmentary alterations, macrocytic anemia, and increased apoptosis in the thymic cortex (Jiang *et al.,* 1998; Perez-Losado *et al.,* 2002). Smuc-null mice have not been generated, but Smuc expression patterns in the developing embryo suggest a role for the protein in the developing skeletal muscle and thymus (Zhuge *et al.,* 2005). Thus, although Snail family members collectively direct morphogenesis during development, they play distinct functional roles in the embryo. Despite evidence that Snail and Slug modulate EMT during embryonic development and tumor progression (Sefton *et al.,* 1998; Moreno-Bueno *et al.,* 2006), little is known about potential functions for these proteins in normal adult tissues.

Our previous studies provided evidence for involvement of Slug in the maintenance of adult epidermis and in cutaneous wound healing (Parent *et al.,* 2004; Savagner *et al.,* 2005). In adult skin, Slug is expressed in hair follicles and the interfollicular epithelium adjacent to hair follicles (Parent *et al.,* 2004). Furthermore, Slug expression is enhanced at the margins of healing wounds *in vitro, ex vivo,* and *in vivo*; expression of Slug coincides with keratinocyte emigration from the wound margin; and keratinocyte outgrowth is impaired in skin explants derived from Slug-null mice (Savagner *et al.,* 2005). Ectopic Slug expression in cultured human keratinocytes causes EMT-like alterations in cell morphology and behavior, including increased cell spreading, desmosomal disruption at wound margins, and accelerated reepithelialization (Savagner *et al.,* 2005). Other investigators have shown that increased Slug expression in cultured keratinocytes also results in decreased expression of the adhesion molecules E-cadherin and integrins $\alpha 3$, $\beta 1$, and $\beta 4$ (Turner *et al.,* 2006). Taken together, these findings suggest that Slug performs important functions in normal

[1]*Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio, USA;* [2]*Division of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, New Mexico, USA;* [3]*College of Medicine and Public Health, The Ohio State University, Columbus, Ohio, USA*

Correspondence: Dr K Newkirk, The University of Tennessee, College of Veterinary Medicine, 2407 River Drive, Room A201, Knoxville, Tennessee 37996-4542, USA. E-mail: knewkirk@utk.edu

Abbreviations: EMT, epithelial-mesenchymal transformation; RT-PCR, reverse transcription-PCR

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adult epidermis and highlight the need to define Slug-controlled transcriptional programs in the skin.

This study identified candidate genes regulated by Slug using comparative gene array analysis of epidermis isolated from Slug-null and wild-type littermates. Differences in gene expression patterns between wild-type and Slug-null epidermis indicated a role for Slug in keratinocyte differentiation, cell adhesion and motility, proliferation and apoptosis, angiogenesis, and response to external stimuli, processes important in epidermal homeostasis, cutaneous wound healing, and skin carcinogenesis. These results support a regulatory role for Slug in normal adult tissue, in addition to its previously reported roles in modulating developmental processes and cancer progression.

RESULTS AND DISCUSSION

Overview of microarray findings

By directly comparing gene expression patterns in the epidermis of Slug knockout and wild-type mice, we were able to gain considerable insight into the potential impact of this transcription factor on skin structure and function. Our novel approach to identifying possible targets of a transcription factor *in vivo* did not rely on commonly employed methods of artificially enhancing or abrogating gene expression in cultured cells. Because RNA was isolated from unperturbed skin of viable mice, the levels of expression determined were very likely to be biologically relevant. Moreover, the use of wild-type and knockout mice on identical genetic backgrounds minimized strain-dependent differences in gene expression. Our RNA isolation technique removed virtually all epidermis from the skin (Figure 1) and yielded large amounts of very high quality RNA (Figure S1). PCR amplification to increase sample size was thus not required, eliminating a potential source of variability and enhancing our ability to generate very consistent replicate microarray assays.

We identified 139 different named genes with significantly increased expression in Slug knockout compared with wild-type epidermis and 109 different genes with significantly decreased expression (Tables S1–S6). These findings indicate that Slug modulates expression of a variety of epidermal genes; however, our studies do not reveal if Slug acts directly or indirectly to regulate expression of these potential target genes. Using quantitative RT-PCR or immunohistochemistry, we verified altered levels of gene expression for a number of these genes (Table 1; Figure 2). Functional classification of genes with significantly altered expression in Slug knockout epidermis supported roles for Slug in development and differentiation, proliferation and apoptosis, adhesion and motility, angiogenesis and, unexpectedly, the response to external stimuli. Morphologic differences between Slug knockout and wild-type epidermis reflected some of the roles indicated for Slug by our microarray results. Our findings suggest that Slug controls the expression of a wide variety of genes important for epidermal homeostasis, cutaneous wound healing, and skin carcinogenesis.

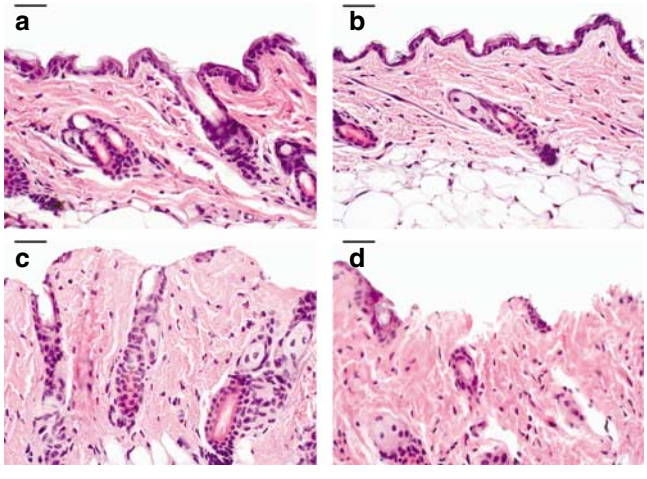

Figure 1. Isolation of epidermal cells from the skin. (**a**) Untreated wild-type and (**b**) Slug knockout skin; (**c**) wild type and (**d**) Slug knockout skin after the epidermis was scraped off, leaving the underlying dermis intact. Bar = 30 μm.

The oPOSSUM program (Ho Sui *et al.*, 2005) was able to utilize information from 73% of the genes with enhanced expression and 62% of the genes with decreased expression in Slug knockout epidermis to identify overrepresented transcription factor binding sites. We used the recommended criterion of a *Z*-value greater than 10 in combination with a Fisher *P*-value of <0.01 to identify overrepresented binding sites likely to be empirically validated. Eleven overrepresented sites were identified in genes expressed at increased levels in Slug knockout epidermis, whereas no overrepresented site was detected in genes with reduced expression (Table 2). Interestingly, there were five zinc-finger transcription factor binding sites among the 11 overrepresented sites in the promoters of genes with enhanced expression. The mammalian Slug/Snail consensus binding site was not among those included in the oPOSSUM database. When the promoter regions of differentially expressed genes were examined specifically for the frequency of the *Drosophila* Snail-binding sites, as a surrogate, Fisher *P*-values were <0.01, but *Z*-scores were <10 for genes with either enhanced or decreased expression in Slug knockout epidermis. Thus, this binding site did not appear likely to account for the overall pattern of altered gene expression in Slug knockout epidermis. For 146 of the 248 genes displaying significant differences in expression between Slug knockout and wild-type epidermis, we were able to examine directly the 1,000 bases upstream from the transcription start site (Tables S1-6). Of these genes, only 80 (55%) contained one or more canonical Snail family binding sites (CAGGTG or CACCTG) (Hemavathy *et al.*, 2000). Of the genes with Slug-binding sites in this region of the promoter, 36 showed decreased and 44 showed increased expression in Slug knockout compared with wild-type epidermis. Taken together, these findings suggest that zinc-finger transcription factors may negatively regulate gene expression in wild-type epidermis, because expression of genes with promoter binding sites for these factors is enhanced in knockout mice lacking Slug. However, neither the Slug nor the Snail transcription factor is specifically implicated in direct control of the differentially expressed genes we identified.

Table 1. Confirmation of microarray findings by qRT-PCR

Gene	Primers	Ratio Slug knockout/wild-type expression by microarray	Ratio Slug knockout/wild-type expression by qRT-PCR[1]	qRT-PCR performance site
Slug	GATGTGCCCTCAGGTTTGAT ACACATTGCCTTGTGTCTGC	0	0	OSU[2]
Gli1	ACTAGGGGGCTACAGGAGGA ACCTGGACCCCTAGCTTCAT	8.5	5.41	OSU
Gli2	CTCAGCCATCTCAGGACACA CAAAGGCTCAGGCTGGATAC	3.4	6.98	OSU
Krt1-8	ATCGAGATCACCACCTACCG TGAAGCCAGGGCTAGTGAGT	35.6	1052.36	OSU
Krt1-18	ABI Mm01601702.g1	22.9	1024.00	UNM[3]
Snf1	ABI Mm00440317.m1	−4.8[4]	−1.15[4]	UNM
Angiomotin	ABI Mm00462731.m1	5.6	8.00	UNM
Tenascin C	ABI Mm00495662.m1	6.1	9.19	UNM

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse transcription-PCR.
[1]All values normalized to GAPDH before ratio calculations.
[2]The Ohio State University.
[3]University of New Mexico.
[4]Negative sign indicates that expression of this gene was decreased in the knockout.

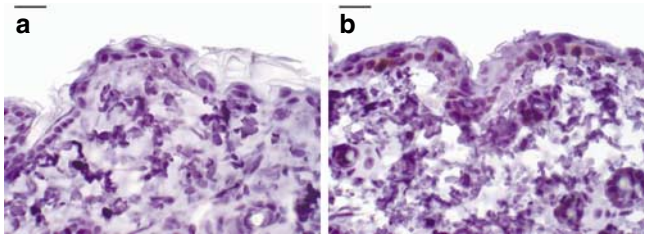

Figure 2. Immunohistochemical detection of keratin 8 in untreated skin. (**a**) There was no keratin 8 staining in the wild-type epidermis, (**b**) whereas scattered cells in the Slug knockout epidermis were immunopositive for keratin 8. Bar = 20 μm.

Development and differentiation

In keeping with the known role of Slug in embryogenesis, the absence of Slug was associated with changes in expression of a variety of other genes related to development and differentiation (Table S1). Widespread alteration in developmental gene expression in Slug knockout epidermis indicated substantial crosstalk among different signaling pathways important during embryogenesis and epidermal differentiation.

Expression of Gli1 and Gli2 was increased in the knockout epidermis by 8.5- and 3.4-fold, respectively; increased expression of these genes in knockout epidermis was confirmed by quantitative RT-PCR (Table 1). Gli1 and Gli2 are zinc-finger transcription factors that mediate sonic hedgehog signaling to regulate cell–cell interactions during embryogenesis (Lewis *et al.,* 2001). Mice transgenic for Gli1 or Gli2 spontaneously develop basal-cell carcinomas (Nilsson *et al.,* 2000; Sheng *et al.,* 2002). Despite increased levels of Gli1 and Gli2, no basal-cell carcinomas occurred either in UV radiation-exposed or unexposed Slug knockout mice during a long-term skin carcinogenesis study (manuscript in review). This may be because expression of patched homolog 1 (Ptch1), a hedgehog receptor, was also increased (1.7-fold) in Slug knockout epidermis. Patched 1 is a tumor suppressor that probably acts as a ''gatekeeper'' to block cell-cycle progression and tumor formation (Adolphe *et al.,* 2006). Loss of patched 1 expression results in constitutive activation of the sonic hedgehog pathway, Gli dysregulation, and basal-cell carcinoma formation (Mancuso *et al.,* 2004; Adolphe *et al.,* 2006). Expression of SNF-like kinase (Snfl1k), which plays a role in muscle differentiation and cardiogenesis (Ruiz *et al.,* 1994; Stephenson *et al.,* 2004), was estimated to be decreased 4.8-fold in Slug knockout epidermis based on microarray findings; RT-PCR demonstrated a similar decrease in Snfl1k expression in the Slug knockout epidermis (Table 1). Snail expression was not altered in the Slug knockout epidermis, indicating that although Slug and Snail may have some redundant functions, Snail expression did not increase to compensate for the absence of Slug expression in unperturbed skin.

A number of genes important in keratinocyte differentiation displayed altered expression in Slug knockout epidermis. One of the genes with very significantly increased expression (more than 35-fold) in the knockout epidermis was keratin 8 (Krt2-8), which encodes an intermediate filament expressed in simple epithelium throughout the body (Owens and Lane, 2003). Enhanced keratin 8 expression in Slug knockout epidermis was consistent with previous studies showing that expression of Slug RNAi in a mammary epithelial cell line increases keratin 8 expression, whereas enhanced expression of Slug in these cells reduces keratin 8 expression (Tripathi *et al.,* 2005a, b). The keratin 8 promoter contains an E-box to which Slug binds to repress transcription (Tripathi *et al.,* 2005a, b). Expression of keratin 18, which is often paired with

Table 2. Over-represented transcription factor binding sites

Expression in Slug knockout mice	Transcription factor	Class	Animal	*Z*-score	Fisher *P*-value
Increased	Nkx2-5	HOMEO	Vertebrate	20.46	1.530^{-3}
	FOXF2	FORKHEAD	Vertebrate	19.07	9.290^{-5}
	SRY	HMG	Vertebrate	18.73	3.537^{-4}
	Hunchback	ZN-FINGER, C_2H_2	Insect	22.03	3.293^{-3}
	Sox5	HMG	Vertebrate	14.81	1.192^{-3}
	Broad-complex 4	ZN-FINGER, C_2H_2	Insect	14.34	8.846^{-3}
	Foxq1	FORKHEAD	Vertebrate	13.74	2.419^{-3}
	Gfi	ZN-FINGER, C_2H_2	Vertebrate	13.08	1.229^{-4}
	Broad-complex 1	ZN-FINGER, C_2H_2	Insect	12.91	7.688^{-3}
	Prrx2	HOMEO	Vertebrate	11.88	1.044^{-3}
	Broad-complex 3	ZN-FINGER, C_2H_2	Insect	10.99	7.315^{-4}
	Snail	ZN-FINGER, C_2H_2	Insect	1.694	3.961^{-3}
Decreased	Snail	ZN-FINGER, C_2H_2	Insect	3.62	7.730^{-3}

keratin 8, was also increased (23-fold) in knockout epidermis; because expression of keratin 8 and 18 was increased to a similar extent, they may also be complexed in knockout epidermis. Enhanced expression of keratins 8 and 18 was confirmed by quantitative RT-PCR (Table 1). RT-PCR indicated substantially higher expression of these keratins than was predicted based on microarray data; this finding is in keeping with other reports that microarray analysis tends to underestimate the extent of overexpression of very highly expressed genes (Yuen *et al.,* 2002). Immunohistochemistry also confirmed increased expression of keratin 8 in Slug knockout epidermis (Figure 2). Keratins 8 and 18 are the first keratins expressed during embryogenesis (Chisholm and Houliston, 1987), and although not normally expressed in the adult epidermis, they are often aberrantly expressed in squamous-cell carcinomas (Larcher *et al.,* 1992; Hendrix *et al.*, 1996; Oshima *et al.,* 1996). Expression of exogenous keratin 8 in a variety of cell lines results in anchorage-independent growth, shortened doubling times, increased invasive and migratory capabilities (Chu *et al.*, 1996), and apoptosis resistance *in vitro* (Gilbert *et al.*, 2001), as well as enhanced metastasis *in vivo* (Raul *et al.*, 2004). Differences in epidermal differentiation indicated by our microarray results were reflected in significantly decreased epidermal thickness in knockout compared with wild-type skin ($7.38\,\mu m \pm 0.99$ *vs* $8.91\,\mu m \pm 1.67$, $P = 0.016$ by the Student's *t*-test).

Proliferation and apoptosis

Slug is known to be antiapoptotic in hematopoietic cells (Inukai *et al.,* 1999; Come *et al.,* 2004), whereas increased Slug expression reduces proliferation in cultured keratinocytes (Bolos *et al.,* 2003; Turner *et al.,* 2006). Proliferation and apoptosis share some upstream signaling pathways, including receptor-tyrosine kinase-dependent mitogen-activated protein kinase pathways known to induce Slug expression (Hudson *et al.,* 2007). A number of significant alterations in components of these pathways were observed in Slug-null epidermis (Table S2).

Expression of cyclins D2 and G2 (Ccnd2, Ccng2), which regulate cell-cycle progression, was decreased by 1.3- and 1.4-fold, respectively, in the Slug knockout epidermis (Table S2), suggesting modestly reduced basal levels of proliferation. This contrasted with findings from a previous study showing decreased keratinocyte proliferation without changes in cyclin D expression in response to enhanced Slug expression (Turner *et al.,* 2006). Moreover, it has been proposed that Snail blocks cell-cycle progression through G_1/S by repressing cyclin D transcription (Vega *et al.*, 2004). Quantitation of Ki-67-positive cells in the epidermis did not reveal a significant difference between wild-type and knockout epidermis (12.04 ± 3.32 *vs* 16.08 ± 7.07, $P = 0.112$ using the Student's *t*-test), indicating no substantial decrease in basal proliferative activity in Slug-null epidermis.

Interactions of regulators of apoptosis are complex and it is the net balance of pro- and anti-apoptotic factors that determines cell fate. Expression of phorbol-12-myristate-13-acetate-induced protein 1 (Pmaip1), also known as Noxa, was increased almost 3-fold in the knockout epidermis. Noxa is a proapoptotic member of the Bcl-2 family; its expression is increased by p53-dependent or -independent mechanisms in response to cellular stress, DNA damage, or growth factor deprivation (Jullig *et al.,* 2006). Noxa interacts with antiapoptotic members of the Bcl-2 family, resulting in release of cytochrome *c* from the mitochondria and subsequent activation of downstream caspases (Jullig *et al.,* 2006). Another key regulator of apoptotic responses, Puma, was previously shown to be regulated by Slug in hematopoietic cells (Wu *et al.,* 2005). Secreted Ly6/Plaur domain containing 1 (Slurp1) was increased 1.8-fold in the knockout epidermis. Slurp1 is a secreted protease that has been shown to play a role in maintaining the integrity of keratinocytes in the epidermis (Mastrangeli *et al.,* 2003), and has proapoptotic effects (Arredondo *et al.,* 2005). Increased expression of both

Noxa and Slurp1 suggested increased susceptibility to apoptosis in the knockout epidermis. This supported previous data suggesting that Slug has antiapoptotic functions (Inukai *et al.,* 1999; Come *et al.,* 2004).

Adhesion and motility

Previous studies have shown a prominent role for Slug and Snail in modulating cell–cell and cell–substrate adhesion by controlling expression of components of adherens junctions, desmosomes, and tight junctions (Savagner *et al.,* 1997, 2005). Thus, it was not surprising that we identified altered expression of such genes in Slug knockout epidermis (Table S3).

Expression of β-catenin in a number of cell types has been shown to be directly repressed by Slug and Snail through binding to an E-box in the promoter. Surprisingly, our study did not identify altered expression of β-catenin in unperturbed Slug knockout epidermis. In Slug knockout epidermis, there was 1.4-fold decreased expression of integrin α6 (Itga6) compared with wild-type epidermis. Integrin α6 pairs with integrin β4 to form part of the hemidesmosome. A recent report described decreased integrin β4 expression in response to enhanced Slug expression, but did not evaluate α6 expression (Turner *et al.,* 2006). Both components of the hemidesmosome, integrins α6 and β4, have E-boxes in their promoter regions (Turner *et al.,* 2006). Thus, the finding that the two integrin genes may be oppositely regulated by Slug was unexpected. Other integrins with E-boxes are α2, α6, αv, α5, α3, β1, and β4. Expression of none of these was significantly altered in Slug knockout epidermis, although expression of integrins α3, β1, and β4 was decreased following induction of Slug expression in human epidermal keratinocytes (Turner *et al.,* 2006).

In addition to differences in cell-associated adhesion molecules, there were marked differences in expression of extracellular matrix components between wild-type and Slug knockout epidermis. Some of these extracellular matrix components play important roles in EMT and have clear implications for the role of Slug in modulating EMT. Expression of both tenascin C (Tnc) and periostin (Postn) was increased by more than 5-fold in Slug knockout epidermis, and enhanced expression of tenascin C was confirmed by RT–PCR (Table 1). Increased expression of tenascin C and periostin in Slug knockout epidermis was unexpected, given that these extracellular matrix components enhance EMT (Maschler *et al.,* 2004; Ogawa *et al.,* 2005; Yan and Shao, 2006), whereas the absence of Slug expression would be expected to suppress EMT.

Slug expression has been associated with enhanced keratinocyte motility, and we detected significant differences between wild-type and Slug knockout epidermis in expression of several genes that modulate actin function. Expression of rho GTPase-activating protein 25 (Arhgap25) was decreased 2-fold in Slug knockout epidermis. Additional actin-modifying proteins with significantly decreased expression in Slug knockout epidermis included calmin (Clmn) and scinderin (Scin), both decreased 1.9-fold. Scinderin is a member of the gelsolin superfamily of actin filament severing proteins; another member of this superfamily, gelsolin, has previously been shown to be regulated by Snail (Tanaka *et al.,* 2006).

Overall, Slug knockout epidermis appeared to have a gene expression profile consistent with a less motile keratinocyte phenotype than wild-type epidermis. It is important to note that, because we examined gene expression in unperturbed skin, we may have failed to detect inducible Slug-controlled genes involved in cell migration during cutaneous wound healing and squamous-cell carcinoma progression.

Angiogenesis

We saw significant differences between wild-type and Slug knockout epidermis in expression levels for a number of modulators of angiogenesis (Table S4). The c-fos induced growth factor (figf), also known as vascular endothelial growth factor D (Vegfd) (Marconcini *et al.,* 1999), was expressed at substantially decreased levels (3.2-fold) in Slug knockout epidermis. Expression of another inducer of angiogenesis, Smoc2 (Rocnik *et al.,* 2006), was also decreased (3.8-fold) in the knockout mice. Reduction of Vegfd and Smoc2 expression suggested a potentially reduced angiogenic response during wound healing or tumor development in Slug knockout mice. These findings were in keeping with a previous report of increased expression of angiogenic mediators such as vascular endothelial growth factor by MDCK cells expressing increased levels of Slug (Moreno-Bueno *et al.,* 2006). On the other hand, expression of two proangiogenic factors was increased in Slug knockout epidermis. Expression of angiomotin (Amot) was increased 5.6-fold in Slug-null epidermis, a finding confirmed by RT–PCR (Table 1), and angiopoietin 1 expression was increased 2.1-fold (Tammela *et al.,* 2005).

Response to external stimuli

The epidermis forms an important barrier between the body and the external environment, thus it might be expected to express a wide variety of molecules involved in protective responses to environmental insults. In keeping with this expectation, we observed altered expression of genes encoding molecules related to inflammatory and immune responses, response to oxidative stress, and metabolism of xenobiotics (Table S5).

Expression of several interleukin receptors differed significantly between Slug knockout and wild-type mice, with more than 3-fold decreased expression of receptors for the proinflammatory cytokines interleukin-20 and -22 (Rich and Kupper, 2001; Boniface *et al.,* 2005). Although our microarray studies were conducted using material highly enriched for keratinocyte RNA, other cell types present in the epidermis contributed at least small amounts of RNA. Such non-keratinocyte cell types included intraepidermal dendritic T cells (γδ T cells) and Langerhans cells. Some of our results may thus be related to differences in gene expression in these immune cells rather than in keratinocytes. For example, expression of the zeta chain of CD3 (Tcrz), an important component of T-cell receptor signaling, was decreased 1.7-fold in Slug knockout epidermis. In addition, expression of

both the γ and δ chains of the T-cell receptor (Tcrg and Tcrd) was decreased approximately 2.3-fold in the Slug knockout epidermis, suggesting that there were fewer $\gamma\delta$ T cells in the knockout than in wild-type epidermis. These $\gamma\delta$ T cells play an important role in innate immunity in the skin, exhibiting anti-tumor and immunoregulatory activity (Girardi, 2006). Mice lacking $\gamma\delta$ T cells have been shown to have increased susceptibility to cutaneous carcinogenesis (Girardi *et al.,* 2001). In keeping with these findings, immunohistochemical staining for CD3 revealed decreased numbers of T cells in the epidermis of Slug knockout and wild-type mice (3.77 ± 2.16 *vs* 4.7 ± 0.86); however, this difference was not significant ($P = 0.199$ by Student's *t*-test).

Miscellaneous

Expression of a number of genes that encode proteins important in membrane transport, intracellular trafficking, and metabolism differed significantly between wild-type and Slug knockout epidermis (Table S6). For most of these genes, the implications of altered expression for epidermal homeostasis, cutaneous wound healing, and skin carcinogenesis was unclear.

Conclusion

Our studies identified a number of biologically relevant putative targets of Slug regulation. In a number of cases, the microarray findings and their functional implications were substantiated by quantitative RT–PCR, immunohistochemistry, or morphometry. Novel features of our approach included comparison of unperturbed epidermis from wild-type and Slug knockout mice, isolation of a relatively pure population of epidermal keratinocytes, isolation of high quality RNA from epidermis, multiple microarray repeats without complementary DNA (cDNA) amplification, thorough statistical analysis to identify differences between wild-type and Slug knockout mice, and functional analysis of gene expression patterns with statistical tests of significance.

Our studies provided valuable insight into the complex patterns of Slug-dependent gene expression in the epidermis and highlighted some of the mechanisms by which Slug may influence epidermal homeostasis, wound healing, and carcinogenesis. It is important to note, however, that our studies revealed the cumulative effects of the absence of Slug during the entire process of epidermal development and differentiation. Thus, alterations in gene expression that we observed may have reflected compensatory changes in genes not directly controlled by Slug. Moreover, structural differences between the skin of wild-type and Slug knockout mice may have altered the relative recovery of RNA from subpopulations of epidermal cells, leading to apparent differences in gene expression.

Likely targets of Slug regulation included both previously and newly identified genes. Developmental genes with altered expression included members of the sonic hedgehog and homeobox pathways, in keeping with the previously identified role of Slug in embryogenesis. The Slug-null epidermis expressed particularly high levels of keratins 8 and 18, indicating a role for Slug in epidermal differentiation. Alterations in expression of genes regulating proliferation and apoptosis, such as growth factors and related genes, cyclins, and Noxa, suggested a role for Slug in keratinocyte turnover. Altered expression of a number of genes encoding cell-associated and extracellular matrix adhesion molecules showed that Slug modulates keratinocyte adhesion in sessile as well as migrating keratinocytes. Our data also indicated a potential role for Slug in cutaneous angiogenesis. A novel role for Slug in modulating the epidermal response to the external environment was suggested by altered basal expression of genes encoding interleukin receptors, T-cell receptors, and a P450 in Slug knockout epidermis. Although several overrepresented binding sites for zinc-finger transcription factors were identified in the promoters of genes with enhanced expression in Slug knockout epidermis, interrogating these promoters for sites of *Drosophila* Snail binding (the closest match to Slug-binding sites) did not reveal enrichment. However, the presence of canonical Slug-binding sites in the promoters of some genes with increased expression in Slug knockout epidermis identified several potential targets of direct Slug repression.

Clearly, further studies are required to validate many of the differentially expressed genes that we identified and to identify those that are direct targets of Slug regulation. One approach would be the study of conditional Slug knockout mice. Such mice are currently being constructed in our laboratory. They will allow us to determine the changing pattern of gene expression over time after abrogation of Slug expression. This will help distinguish those genes under the direct control of Slug from those with secondarily altered expression. To validate direct transcriptional control of specific genes by Slug will necessitate chromatin immunoprecipitation studies. To date, such studies *in vivo* have been hindered by the lack of a reliable immunoprecipitating Slug antibody, a deficiency that will hopefully be remedied in the near future.

MATERIALS AND METHODS

Mice

The mice employed in these studies were generated by Dr Thomas Gridley (Jackson Laboratory, Bar Harbor, ME) (Jiang *et al.,* 1998). In these mice, the zinc-finger region of the Slug gene has been replaced by a β-galactosidase gene, resulting in the formation of a Slug-β–galactosidase fusion protein. The Slug portion of this protein is non-functional because it lacks the zinc-finger region; however the β-galactosidase portion is fully functional. The mice were originally produced on a mixed C57/BL6 $\times$ 129S1/SvImJ background (Jiang *et al.,* 1998), but the Slug knockout allele was subsequently transferred to an inbred 129S1/SvImJ background (Dr Thomas Gridley, personal communication); mice are propagated at the Ohio State University by mating inbred 129 heterozygotes, thus generating wild-type, Slug knockout, and heterozygous knockout siblings.

For each microarray analysis, two wild-type and two homozygous Slug knockout 12-week-old female mice were employed. The mice were killed by carbon dioxide inhalation, shaved with electric clippers, and depilated with Nair. Dorsal skin was removed and immediately frozen as 3×4 cm strips in liquid nitrogen for later RNA isolation. Additional skin samples were fixed in formalin for

routine histopathology. All of the mice were in telogen, as evidenced grossly and confirmed histologically.

All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC).

Microarray analysis

The epidermis was vigorously scraped from frozen skin samples, using a scalpel blade. Samples were placed in Trizol (Invitrogen, Carlsbad, CA), then homogenized and processed as recommended by the supplier. This technique isolates primarily keratinocytes with small numbers of Langerhans cells and intraepidermal dendritic cells that are resident in the epidermis. Wild-type and knockout samples were processed simultaneously. Skin was fixed in formalin after scraping and examined histologically to confirm that the epidermis was completely removed (Figure 1).

RNA samples were purified using an RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quality was verified by the Microarray Unit (MAU) at The Ohio State University Comprehensive Cancer Center (OSUCCC), which performed subsequent microarray analysis. High-quality RNA was consistently isolated using this technique (Figure 2). For each microarray analysis, two wild-type and two knockout total RNA samples were pooled based on molar quantity and were submitted to the OSUCCC-MAU for further processing to ensure highly standardized techniques. RT was performed using 8 μg total RNA, a T7-(dT)24 primer, and Superscript II reverse transcriptase (Invitrogen). Second-strand cDNA synthesis was performed using *Escherichia coli* DNA polymerase I, DNA ligase, and RNase H (Invitrogen). The resulting double-stranded cDNA was cleaned using the cDNA GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA). *In vitro* transcription was carried out using biotin-labeled ribonucleotides and T7 RNA polymerase. The resulting cRNA was purified using the IVT cRNA GeneChip Sample Cleanup Module (Affymetrix). After the labeled cRNA was fragmented at 95°C for 35 minutes, hybridization was performed in a buffer consisting of 150 μl of hybridization buffer (Affymetrix), 30 μg of herring sperm DNA, 60 μg of acetylated BSA, 40 μl of cRNA, and biotinylated control oligonucleotides at 45°C for 16 hours with rotation to the Gene Chip Mouse Genome 430 2.0 Array. After being washed in an Affymetrix Fluidics station, the arrays were stained with streptavidin R-phycoerythrin (Vector, Burlingame, CA) and scanned with an HP-laser scanner (Affymetrix) to produce data files containing raw pixel intensities. The entire microarray analysis was repeated twice more for a total of three independent analyses. Thus, our data represented RNA from six wild-type and six knockout mice.

Data analysis

The raw intensity files from the scanner were initially processed with the program MicroArray Suite, version 5 (MAS5) from Affymetrix. This software was used to generate probe-pair level data (CEL files) and probe-set level data (CHP files). The CEL files from MAS5 were processed with RMA-Express version 0.3 (Bolstad BM, University of California, Berkeley, CA) to obtain background-subtracted and quantile-normalized expression values for each probe. The CHP files generated were of two types. The first type of CHP file (CHP-1) was a single-chip analysis yielding present, marginal, or absent calls for all probe sets. The second type of CHP file (CHP-2) was a two-chip comparative analysis which compared all pair-wise combinations of each of the three wild-type and knockout replicates and yielded increased, decreased, or no Change calls for each probe set. The call results in these two files were used to filter the list of potential probes. To eliminate probes showing little change across all of the arrays, the list of probes contained on the microarray chips was subjected to two levels of filtration based on the calls in the CHP files. The first elimination step used the single-chip analyses to eliminate any probe that did not have at least two present calls in either the wild-type or Slug knockout group. Next, the comparative CHP files were used to eliminate any probes that did not have at least six increased or six decreased calls out of the nine pair-wise comparisons. The probes passing this second filter became the final list for the RMA data files. Filtered RMA expression values were analyzed with a two-sample, equal variance *t*-test comparing Slug knockout to wild-type signals. *P*-values less than or equal to 0.05 were considered significant. Bonferroni's correction (Motulsky, 1995) was applied to each *P*-value to obtain an adjusted *P*-value to identify differentially expressed probes with high statistical significance.

With the help of DAVID Bioinformatic Resources 2006 (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (Dennis *et al.,* 2003), Affymetrix database, and relevant publications, the genes that were significantly altered based on the basic *t*-test were categorized by known function. Unnamed genes were excluded from this categorization. Over-represented transcription factor binding sites were identified in the promoters of differentially expressed genes using oPOSSUM (Ho Sui *et al.*, 2005). This program compares statistically the frequency of binding sites in a given set of genes with the frequency of those sites in a reference gene set containing 2.4×10^6 highly conserved predicted binding sites. Results are reported both as a *Z*-score and a one-tailed Fisher exact probability *P*-value. For our studies, genes with increased and decreased expression in Slug knockout mice were examined separately, using the most stringent predefined values for binding site detection (minimum 70% conservation threshold, with a position-specific scoring matrix match score of 85% and a promoter region defined as extending 2,000 bases upstream of the transcription start site). Because the *Drosophila* but not the mammalian Snail site was included among the transcription factor binding sites considered by oPOSSUM, we examined genes for all insect transcription factor binding sites.

Quantitative RT–PCR

Total RNA from the Slug knockout and wild-type mice that remained following microarray analysis was used to confirm microarray results by real-time quantitative PCR (Table 1). For analyses performed at The Ohio State University (Slug, keratin 8, Gli1, and Gli2), 5 μg pooled total RNA was treated with DNaseI (Ambion, Austin, TX), and cDNA was produced by RT of 500 ng of this RNA using Superscript II (Invitrogen) and oligo(dT) primers, as directed by the manufacturer. Quantitative RT–PCR was performed using the primer sets shown in Table 1. The Brilliant SYBR Green QPCR mix (Stratagene, Cedar Creek, TX) was used as directed with 100 nM of each primer in an MX3000P Real-Time PCR System (Stratagene). Fifty cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (30 seconds) were performed. RNA concentrations were calculated using the LinReg PCR program which uses four points in the best linear region of amplification to determine starting mRNA concentration and PCR efficiency for each sample (Ramakers *et al.,* 2003).

Glyceraldehyde 3-phosphate dehydrogenase was used as an internal standard to account for efficiency of RT and amplification. Expression values for each primer set were normalized to glyceraldehyde 3-phosphate dehydrogenase values. Reactions were performed in triplicate.

For analyses performed at the University of New Mexico, College of Pharmacy (keratin 18, Snf1, angiomotin, and tenascin C), cDNA was made using the ABI High Capacity cDNA archive kit as directed (Applied Biosystems, Foster City, CA). Taqman master mix, primers, and probes (Table 1) were obtained from Applied Biosystems and used as directed. Samples were run and analyzed using the 7900HT Real-Time PCR System (Applied Biosystems). The ΔC_t was determined by subtracting the average C_t for glyceraldehyde 3-phosphate dehydrogenase (normalizing gene) from the average of the C_t for the gene of interest. The relative expression in knockout *versus* wild-type samples was then determined using the $\Delta\Delta C_t$ method. The efficiency of amplification of all primer and probe sets was verified to be near 100%.

Histology and immunohistochemistry

Skin from the six wild-type and six Slug knockout mice used for RNA isolation was fixed in neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and mounted on glass slides; these slides were stained with hematoxylin and eosin or used for immunohistochemistry. Specimens for immunohistochemistry were deparaffinized and dehydrated, then pretreated with DakoCytomation target retrieval solution (Dako, Carpinteria, CA) using the Biocare Digital Decloaking Chamber (Biocare, Concord, CA) and heated to 125°C for 30 seconds for antigen retrieval. Peroxidase blocking was performed with a 3% peroxidase solution for 5 minutes. A protein block (DakoCytomation Serum-free Protein Block; Dako) was applied for 10 minutes. Staining was carried out with the following primary antibodies: a monoclonal IgG2a, kappa light chain rat anti-mouse keratin 8 (Developmental Studies Hybridoma Bank, University of Iowa Department of Biological Sciences, Ames, IA) diluted 1:250, CD3 (Dako, cat. no. A0452) diluted 1:100, and Ki-67 (Dako, cat. no. M7249) diluted 1:100. Primary antibodies were diluted in DakoCytomation Antibody Diluent (Dako) and applied to the slides for 30 minutes. Sections were incubated with secondary antibodies (biotinylated rabbit anti-rat and biotinylated rabbit anti-rat mouse adsorbed (Vector) diluted 1:200 in Serum-free Protein Block) for 30 minutes, followed by a 30-minute incubation with ABC reagent (Vector R.T.U. Vectastain Elite ABC, Burlingame, CA), a 5-minute incubation with chromagen (DakoCytomation Liquid DAB Substrate; Dako), and hematoxylin counterstaining. Rinses were performed using DakoCytomation Wash Buffer (Dako). The slides were then dehydrated and cover-slipped.

Epidermal thickness was determined using an ocular micrometer. Six different fields were measured at a magnification of $\times 600$, and then the values from each mouse were averaged.

CD3-positive cells had strong cytoplasmic staining. CD3-positive cells were counted in the epidermis of six different $\times 400$ fields. In each of these fields, the total number of epidermal cells was also counted, and the number of CD3-positive epidermal cells was expressed as a percentage of the total epidermal cells. These numbers were averaged for each genotype and then compared. Ki-67-positive cells have strong nuclear staining and were quantified as described for CD3 staining. Results were compared using the Student's *t*-test; *P*-values of <0.05 were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Altered expression of development and differentiation-related genes in Slug knockout epidermis.

Table S2. Altered expression of proliferation and apoptosis-related genes in Slug knockout epidermis.

Table S3. Altered expression of adhesion and motility-related genes in Slug knockout epidermis.

Table S4. Altered expression of angiogenesis-related genes in Slug knockout epidermis.

Table S5. Altered expression of genes related to responses to external stimuli in Slug knockout epidermis.

Table S6. Miscellaneous genes with altered expression in Slug knockout epidermis.

Figure S1. Isolation of epidermal cells from the skin.

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