Long Noncoding RNA: Significance and Potential in Skin Biology

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Over the past few years, advances in genome analyses have identified an emerging class of noncoding RNAs that play critical roles in the regulation of gene expression and epigenetic reprogramming. Given their transcriptional pervasiveness, the potential for these intriguing macromolecules to integrate a myriad of external cellular cues with nuclear responses has become increasingly apparent. Recent studies have implicated noncoding RNAs in epidermal development and keratinocyte differentiation, but the complexity of multilevel regulation of transcriptional programs involved in these processes remains ill defined. In this review, we discuss the relevance of noncoding RNA in normal skin development, their involvement in cutaneous malignancies, and their role in the regulation of adult stem-cell maintenance in stratified epithelial tissues. Furthermore, we provide additional examples highlighting the ubiquity of noncoding RNAs in diverse human diseases.

AN EMBARRASSMENT OF RICHES?

For most of the past few decades, the focus of molecular biologists has centered around protein-coding genes in the genome, although they comprised <2% of the human genome sequence (Lander et al. 2001; Venter et al. 2001). RNA was often regarded as a simple intermediary between DNA and protein. More recently, with the advent of large-scale analyses of mammalian transcriptomes (Claverie 2005; Kapranov et al. 2007; Mercer et al. 2009), scientists have come to appreciate a new class of transcripts, noncoding RNAs (ncRNAs), that are pervasively transcribed in the genome. Why are

they so pervasive and do they have function? Long thought to be transcriptional “noise,” these RNAs have now been increasingly implicated to play functional roles in gene regulation (Wang and Chang 2011). In contrast to other better characterized, small ncRNAs such as microRNAs (miRNAs) and Piwi-interacting RNAs, which are highly conserved and involved in transcriptional and posttranscriptional gene silencing through specific base pairing with their targets (He and Hannon 2004; Mendell 2005; Aravin et al. 2007; Brennecke et al. 2007), long noncoding RNAs (lncRNAs), which are arbitrarily defined as transcribed RNA molecules >200 nucleotides in length, are poorly con-

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served and regulate gene expression by diverse mechanisms that are not yet fully understood (Bernstein and Allis 2005; Bracken and Helin 2009; Faghihi and Wahlestedt 2009; Mercer et al. 2009; Whitehead et al. 2009; Wilusz et al. 2009; Wang and Chang 2011).

Although only a handful of functional lncRNAs have been well characterized to date, recent work suggests that lncRNAs are at the heart of diverse biological processes such as imprinting (Nagano and Fraser 2009), enhancer functions (Ho et al. 2006; Kim et al. 2007; Ørom et al.

2010a), X-chromosome inactivation (Lee 2009), and chromatin structure (Rinn et al. 2007; Wang et al. 2011). They also appear to be critical for normal development and have been found to be dysregulated in many diseases such as cancer (Fig. 1) (Ørom et al. 2010b).

Given the large number of lncRNAs whose functions are now only beginning to be elucidated, there is clear potential for widespread regulation and/or modulation of gene expression. Furthermore, it is easy to imagine lncRNAs playing major roles in determining the epige-

Figure 1. Functional schematic of lncRNAs. lncRNAs may function by partnering with small molecule regulators and/or mediators (such as transcription factors, chromatin modification enzymes, or DNA-binding proteins) or by stabilizing RNAs through posttranscriptional mechanisms to bring about gene expression changes via histone modifications and chromatin folding. This ultimately results in downstream regulation of disparate processes including tumorigenesis, embryonic development, wound healing, and cellular differentiation.

netic status and transcriptional network in any given cell type, and that they provide a means to integrate external differentiation cues with dynamic nuclear responses through the regulation of a metastable epigenome. It is clear that the transmission of genetic information involves a dialog between DNA and RNA, mediated by ncRNAs; the input of these nonprotein-coding RNAs is likely to be essential in shaping transcriptional and, consequently, genomic output. Moreover, interruption of RNA-mediated feedback is sure to perturb gene expression programs and bring about human disease.

What are the roles of these lncRNAs in skin biology? During the last few years, work from multiple groups has shown that epigenetic mechanisms are involved in the control of epidermal development, keratinocyte differentiation, and melanocyte functions (Millington 2008). However, many aspects of the epigenetic control of gene expression programs in skin remain to be elucidated—for example, the association of ncRNAs with functional activity of the various cutaneous cell types, the fundamental significance of these epigenetic mechanisms during skin development, differentiation, aging, and regeneration, and the still unknown complexity of multilevel regulation of gene expression programs in healthy and diseased skin are all poorly understood.

CURRENT EXAMPLES OF lncRNAs WITH IMPORTANT BIOLOGICAL FUNCTIONS

The relevance of the noncoding genome to human disease has mainly been studied in the context of widespread disruption of miRNA expression and function that is seen in human cancers (Esquela-Kerscher and Slack 2006; Hammond 2007; Croce 2009; Nicoloso et al. 2009). Recent studies, however, have begun to shed light on the manifold roles lncRNAs may play in diverse biological processes. Focusing specifically on skin, these include HOX gene regulation, development of melanoma, and epidermal differentiation (Table 1). In addition, lncRNAs have also been shown to be involved in regulating cancer metastasis and to be associated with a variety of genetic syndromes.

HOTAIR Brings lncRNAs to the Forefront

It has long been thought that HOX genes, key players in embryonic development, are regulated by *cis*-acting ncRNAs (Brock et al. 2009). Rinn and colleagues, in 2007, were able to systematically identify hundreds of ncRNAs transcribed from the HOX gene cluster of 11 human fibroblast cell lines isolated from distinct positions along the anteroposterior and proximodistal body axes (Rinn et al. 2007). Interestingly, the ncRNAs were found to be differentially expressed in the fibroblasts according to their original position along the body axis. They further characterized one of the lncRNAs in the HOXC cluster, called HOTAIR, and made the intriguing discovery that it acts in *trans* to regulate another HOX gene cluster several chromosomes away. Knockdown of HOTAIR showed no changes in the HOXC cluster in which it was transcribed, but instead resulted in a loss of transcriptional repression from a 40-kb region of the HOXD cluster (Rinn et al. 2007). In addition, the repressive mark, trimethylated histone H3K27, and the epigenetic regulatory complex that produces this mark, the polycomb complex PRC2, were no longer present at that particular region of the HOXD cluster when HOTAIR was depleted. Pull-down experiments of PRC2 components showed a direct and specific interaction with HOTAIR lncRNA (Rinn et al. 2007). These findings raise intriguing questions about how lncRNAs function and provides a glimpse of the functional complexity of this class of regulatory RNAs.

More recently, expression of HOTAIR has been associated with cancer metastasis (Gupta et al. 2010). Elevated expression of HOTAIR is observed in primary and metastatic breast cancer, accompanied by an altered chromatin state; PRC2 is recruited to almost 1000 genes that normally do not bind to PRC2 in epithelial cells, resulting in the down-regulation of multiple metastasis suppressor genes, including HOXD10. Intriguingly, the altered PRC2 occupancy profile induced by HOTAIR in breast epithelial cell resembles the PRC2 occupancy profile in distal fibroblasts in which HOTAIR is normally expressed (Gupta et al. 2010). Con-

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Table 1. Representative lncRNAs with epidermal phenotypes and their proposed mechanisms of action

lncRNA	Method of discovery	Expression	Proposed role	Interacting molecule(s)
ANCR (antidifferentiation ncRNA)	RNA sequencing	Undifferentiated keratinocytes, adipocyte, osteoblasts (Kretz et al. 2012)	Suppression of epidermal differentiation in progenitor compartment (Kretz et al. 2012)	May associate with EZH2, guides PRC2 to suppress differentiation genes (Zhu and Xu 2013)
TINCR (terminal differentiation–induced ncRNA)	RNA sequencing	Up-regulated in differentiating keratinocytes (Kretz et al. 2012)	Regulates genes required for terminal differentiation; down-regulation noted in squamous-cell carcinoma (Kretz et al. 2012)	Associates with differentiation messenger RNAs (mRNAs)/ STAU1, increases mRNA stability in differentiated epidermis
U1 RNA (small nuclear RNA)	RNA sequencing keratinocytes versus nonirradiated	Up-regulated in UVB-irradiated keratinocytes (Bernard et al. 2012)	Promotes repair and maintenance of epidermal permeability (Bernard et al. 2012)	Interacts with TLR3 activating innate immune response (Bernard et al. 2012)
PRINS (psoriasis susceptibility–related RNA gene induced by stress)	Differential expression between unaffected epidermis in psoriasis patients versus normal patients (Sonkoly et al. 2005)	Increased in psoriatic epidermis (Sonkoly et al. 2005)	Involved in cellular stress response	Regulates GIP3, which has antiapoptotic effects in keratinocytes, down-regulates GIP3 in psoriasis (Szegedi et al. 2010)
BANCR (BRAF-activated nonprotein-coding RNA)	RNA sequencing normal epidermal melanocytes versus melanocytes with BRAFV600E ectopic expression versus melanoma with BRAF mutation (Flockhart et al. 2012)	Overexpression in melanoma specimens	Regulates/promotes melanoma cell motility	Unknown
SPRY4-IT1 (intron in SPRY4 gene)	ncRNA microarray comparing melanoma with normal melanocytes and keratinocytes (Khaitan et al. 2011)	Localized in cytoplasm of melanoma cells (Khaitan et al. 2011)	Enhances cell viability; inhibits apoptosis in melanoma cell line, may promote cell motility/invasion (Khaitan et al. 2011)	Unknown

versely, depletion of HOTAIR from cancer cells led to reduced invasiveness (Gupta et al. 2010), suggesting that lncRNA-mediated targeting of PRC2 complexes may be a critical event during breast tumorigenesis. What is perhaps most interesting (and potentially more clinically significant) is the finding that not only is HOTAIR highly induced (up to 2000-fold) in metastatic breast cancer samples, high expression of HOTAIR in primary breast tumors is a powerful predictor of eventual metastasis and death, independent of known clinical/pathologic risk factors (Gupta et al. 2010). The implication of this set of findings is that lncRNAs, such as HOTAIR, can and often do alter and regulate epigenetic states in cells to affect phenotypic changes by targeting of chromatin-modifying complex occupancy/localization/enzymatic activity in *trans*.

HOTTIP Representing Tip of the Iceberg in Mechanisms of Locus Control

The same group that identified HOTAIR added another twist to the lncRNA story by characterizing another lncRNA from the human HoxA cluster that was appropriately named HOTTIP, for HoxA transcript at the distal tip (Wang et al. 2011). In contrast to HOTAIR, HOTTIP was shown to be important for the active expression of an entire cluster of HoxA genes in *cis* by serving as a key intermediate that transmits information from higher-order chromosomal looping into chromatin modifications (Wang et al. 2011). The investigators further show that HOTTIP binds specifically to WDR5, part of the histone methyltransferase MLL1 complex. Artificial tethering of HOTTIP RNA upstream of a luciferase reporter successfully boosted reporter transcription in the presence of WDR5, confirming the importance of the HOTTIP RNA itself in transcriptional activation, rather than merely the act of transcription through the HOTTIP genomic locus (Wang et al. 2011). These data suggest that HOTTIP, by virtue of its location owing to chromosomal looping, can coordinately activate the HoxA genes through recruitment of histone-modifying enzymes, a process that is reminiscent of the phenomenon

termed locus control. It remains to be seen whether lncRNAs such as HOTTIP are as prevalent and significant in gene activation as their known roles in gene silencing.

lncRNAs with Critical Roles in Epidermal Differentiation and Cancer

Of specific interest to skin biologists is the explosion of progress that has been made over the past few years uncovering the widespread involvement of lncRNAs in seemingly every important process in cutaneous biology. A few such examples will be discussed in depth.

One of the prototypes of epidermal lncRNAs is ANCR, which was recently shown to maintain the epidermal progenitor state (Kretz et al. 2012), providing a glimpse into how these RNAs can act to maintain the undifferentiated state in somatic tissue progenitor cells. Using transcriptome sequencing and tiling arrays to compare lncRNA expression in human keratinocytes during calcium-induced differentiation, Kretz and colleagues (2013) identified an lncRNA that was down-regulated during epidermal differentiation; expression of the RNA was also found to be decreased in other terminally differentiated cell types including osteoblasts and adipocytes. ANCR depletion by RNA interference led to rapid differentiation gene induction in cultured keratinocytes as well as in regenerated, organotypic epidermal tissue, a model that has been used to faithfully recapitulate the histology, structure, and gene expression of human epidermis (Ridky et al. 2010). Interestingly, ANCR depletion resulted in differentiation protein expression in the epidermal basal layer from which they are normally excluded. Thus, ANCR is required to enforce the undifferentiated cell state within the epidermis. It is likely that ANCR, similar to other lncRNAs such as HOTAIR and HOTTIP, exerts its effects through binding to a protein partner. Understanding the intricate relationship between the lncRNA and its binding protein will undoubtedly uncover additional novel mechanisms in gene regulatory pathways.

In addition to ANCR, Kretz and colleagues (2013) also recently uncovered a novel lncRNA

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with significant functional impact on homeostasis and differentiation of mature epidermal tissue. Using cultured keratinocytes and high-throughput full transcriptome sequencing, they found an lncRNA, TINCR that was required for maintaining high mRNA abundance of key differentiation genes such as filaggrin, loricrin, and members of the arachidonate lipoxygenases family ALOXE3 and ALOX12B, many of which are mutated in human skin diseases. TINCR-deficient epidermis lacked terminal differentiation ultrastructures, including keratohyalin granules and intact lamellar bodies.

Surprisingly, it turns out that TINCR controls human epidermal differentiation by a post-transcriptional mechanism. Genome-scale RNA interactome analysis revealed that TINCR interacts with a suite of differentiation mRNAs and a high-throughput screen to analyze TINCR-protein binding revealed direct binding of TINCR RNA to the Staufen1 (STAU1) protein (Kretz et al. 2013). Interestingly, STAU1-deficient tissue recapitulated the impaired epidermal differentiation seen with TINCR depletion. Furthermore, the TINCR/STAU1 complex appears to mediate stabilization of differentiation mRNAs, such as keratin 80, an important structural protein in keratinocyte epithelium. Taken together, these data point to TINCR as a key lncRNA required for epithelial differentiation through inducible lncRNA binding to differentiation mRNAs to ensure their expression. Therefore, this supports a potentially important role for lncRNAs and the proteins and mRNAs that interact with them in the control of somatic tissue differentiation.

lncRNAs have also been implicated as important players in cutaneous malignancies. By applying sequence-tagged site real-time PCR-based gene dose mapping to a French melanoma-neural system tumor syndrome family, Pasamant et al. (2007) identified a major lncRNA candidate, ANRIL (antisense noncoding RNA in the INK4 locus), located in the p15/CDKN2B-p16/CDKN2A-p14/ARF gene cluster and associated with melanoma. The investigators found coordinated transcriptional regulation of ANRIL and p14/ARF (as well as p16/CDKN2A and p15/CDKN2B to a lesser extent)

in both normal human tissue and human breast tumors. Using an ncRNA microarray, Perera and his colleagues compared lncRNAs in several melanoma cell lines, melanocytes and keratinocyte controls, as well as in different human patient samples (Khaitan et al. 2011), and found that a number of lncRNAs are differentially expressed in melanoma cell lines in comparison to melanocytes and keratinocyte controls. One lncRNA in particular, SPRY4-IT1, was found to be elevated in melanoma cells in which it promotes cellular survival and invasion. Knock-down results in defects in cell growth, differentiation, and higher rates of apoptosis in melanoma cell lines (Khaitan et al. 2011). Thus, increased expression of lncRNAs such as SPRY4-IT1 may play an important role in the molecular underpinnings of human melanoma and perhaps can even serve as an early biomarker for its detection. The molecular mechanisms by which SPRY4-IT1 acts to affect melanoma progression and metastasis remain to be determined.

Taking advantage of next-generation sequencing technologies, Flockhart and colleagues (2012) compared the transcriptional profiles of wild-type primary human melanocytes with those carrying the BRAF (V600E) mutation and identified another candidate lncRNA involved in the migration of melanoma cells in vitro. Termed BANCER, this RNA is also highly expressed and up-regulated in primary human melanomas with the BRAF (V600E) mutation. The molecular mechanisms by which BANCER acts to affect melanoma progression and metastasis remain to be determined. Similarly, Lee and colleagues, using a genome-wide transcriptome profiling approach via RNA-sequencing in patient-matched populations of malignant versus nonmalignant CD4⁺ T cells, have identified a group of Sezary cell-associated transcripts (SeCATs) of yet unknown function that may be predictors of disease prognosis (Lee et al. 2012).

Involvement of lncRNAs in Genetic Syndromes with Cutaneous Manifestations

Multiple lines of evidence increasingly link mutations and dysregulation of lncRNAs to di-

verse human diseases. Alterations in the primary structure, secondary structure, and expression levels of lncRNAs as well as their cognate RNA-binding proteins underlie diseases ranging from neurodegeneration to cancer (Wapinski and Chang 2011). A few examples include H19 and KCNQ1OT1 in Beckwith–Wiedemann syndrome, H19 in Silver–Russell syndrome, and NESP antisense in McCune–Albright syndrome (Eggermann 2009).

Beckwith–Wiedemann syndrome, a loss-of-imprinting pediatric overgrowth disorder involving congenital abdominal wall defects, macroglossia, and gigantism, has been reported to have an incidence of one in approximately 14,000 births (Choufani et al. 2010). Recent studies have described aberrant expression of KCNQ1OT1, a 91-kb lncRNA localized to the nucleolus, to be involved with this syndrome (Pandey et al. 2008; Chiesa et al. 2012). KCNQ1OT1 resides in the KCNQ1 locus that contains 8–10 protein-coding genes expressed from the maternal allele along with the paternally expressed lncRNA (Kanduri 2011). Investigations have identified interactions between KCNQ1OT1 and both histone methyltransferase G9a, which dimethylates H3K9, and PRC2, resulting in transcriptional silencing of the KCNQ1 locus (Pandey et al. 2008). An 890-bp silencing domain on the 5' end of KCNQ1OT1 has also been shown to facilitate the interaction of DNA methyltransferase 1 with chromatin, leading to changes in methylation patterns at the KvDMR1 and H19/IGF2 clusters (Lee et al. 1999; Mohammad et al. 2012; Robbins et al. 2012). Subsequent hypermethylation of the H19 promoter region has been reported to be a major cause for gigantism (Blick et al. 2006). Collectively, such changes to the epigenetic landscape have thus been associated with misregulation of imprinted loci and linked to the development of Beckwith–Wiedemann syndrome.

In contrast to Beckwith–Wiedemann syndrome, hypomethylation of the paternal H19 promoter region has instead been reported in a series of patients with growth retardation and asymmetric development seen in the full spectrum of Silver–Russell syndrome (Blick et al. 2006). Classically, patients with Silver–Russell

syndrome show intrauterine growth retardation, poor postnatal growth, short stature, and a triangular, asymmetric face, with long eyelashes, thin lips, and mild retrognathia (Price et al. 1999). Although our understanding of H19 continues to evolve, this 2.3-kb lncRNA has been shown to also contain an miRNA (miR-675) in exon one (Cai and Cullen 2007). Both sense and antisense transcripts from the H19 locus have been identified and found to bind PRC2, thereby acting at the level of transcription to regulate Igf2 (Zhao et al. 2010). Furthermore, H19 has also been shown to bind other Igf2 mRNA binding-protein family members, thereby functionally competing for Igf2 mRNA as a mechanism for posttranscriptional regulation (Runge et al. 2000). As recent studies have now begun to suggest, an association between Silver–Russell syndrome and assisted reproductive technologies such as in vitro fertilization, the specter of epimutations at paternal loci, has been raised with this process (Butler 2009; Chopra et al. 2010).

McCune–Albright syndrome, a genetic disorder with polyostotic fibrous dysplasia, endocrine hyperfunction, and unilateral café-au-lait spots, has been described to arise from a postzygotic mutation in the gene GNAS1 that prevents down-regulation of cAMP signaling (Lietman et al. 2007). The GNAS locus is a complex region containing NESP and GNASX1, proteins with unknown functions expressed in neuroendocrine tissues, along with the G protein α -subunit GNAS (Wroe et al. 2000). Interestingly, strand-specific analysis has now confirmed the existence of a paternally expressed NESP antisense transcript that may play a role in regulation of the GNAS cluster (Williamson et al. 2011). NESP antisense is thought to modulate demethylation of H3K4me3 and deplete H3K36me3, resulting in repression in *cis* of its sense counterpart NESP (Williamson et al. 2011). Because NESP forms a single transcription unit with GNASX1 and GNAS, these findings thus have potential implications for epigenetic silencing by lncRNA in the development of McCune–Albright syndrome.

With these studies highlighting the involvement of lncRNAs in Beckwith–Wiedemann,

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Silver–Russell, and McCune–Albright syndromes, it would therefore not be surprising to find additional lncRNAs that may play significant functional roles in a multitude of other pathologies. Indeed, investigations have begun to characterize various lncRNAs in the development of atherosclerosis, Alzheimer’s disease, and transient neonatal diabetes (Temple 2002; Faghihi et al. 2008; Burd et al. 2010; Esteller 2011). And over the next several years, undoubtedly more lncRNAs will continue to be characterized, many of which may be disrupted in other nontumoral disorders.

CONCLUSION AND MAJOR OPEN QUESTIONS

Of course, there is also considerable interest and potential in the application of similar transcriptome interrogation approaches to the study of other types of dermatologic disorders, such as other malignant and benign neoplasms, mosaic disorders, genodermatoses, and inflammatory and even autoimmune processes. Because RNA, like DNA, is also inherently sequence-specific and transcribed in a developmentally specific manner, RNA (and by extension lncRNAs) would also be an ideal regulator of spatial and temporal specificity during both normal development and disease. For instance, the Hox lncRNA HOTTIP is transcribed in one location in the genome, at approximately fewer than one copy per cell on average (Wang et al. 2011), therefore, it is uniquely positioned to modulate specific chromatin modifiers to that location. One can certainly imagine numerous instances in which the interactions between the lncRNAs and their protein and/or nucleic acid partners in specific cell types at specific times synergize to bring about particular cutaneous phenotypes in the organism.

Another broad area of current and future exploration is that of lncRNAs as drug targets. As more lncRNAs are unraveled and their roles dissected, it is becoming increasingly evident that the involvement of these molecules in cancer is much more extensive than initially thought. Several miRNA-expression analyses in both hematological malignancies and solid

tumors have shown that, aside from significant differences in expression between tumor and normal states, distinct tumor-specific miRNA signatures exist—perhaps this would be the case for lncRNAs as well. lncRNAs and the protein machineries that are involved in their biogenesis or activity are attractive targets of novel therapeutic approaches. Thus far, most work in this area is in the context of the role of miRNAs in cancer (Stenvang et al. 2008). Better characterization of the lncRNA-protein “interactome” will undoubtedly lead to new molecular insights and hereto untapped opportunities for researchers and clinicians to modulate the genome at the epigenetic level to treat conditions such as cancer. In similar fashion, dynamic expression profiles for specific miRNAs have been shown to exist at various phases of wound healing that may guide angiogenesis (Shilo et al. 2007). Parallel regulation of lncRNAs during wound regeneration would likely contribute to this process also, which may provide novel targets for the development of therapeutics aimed at the treatment of chronic nonhealing wounds. As far as the future impact and success of the various approaches are concerned, these are still in their incipient stages, but the potential is enormous.

Because of their extremely precise regulation, depending on factors like cellular state, tissue type, or disease, lncRNAs also represent a novel class of potent biomarker candidates. Additionally, because of the relative novelty of the research area and the large number of lncRNAs that are being identified on an ongoing basis, there are near-infinite combinations of mechanisms at the molecular level that offer broad therapeutic possibilities.

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