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Hairless and the polyamine putrescine form a negative regulatory loop in the epidermis

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Abstract

Hairless (HR) is a nuclear protein with co-repressor activity that is highly expressed in the skin and hair follicle. Mutations in *Hairless* lead to hair loss accompanied by the appearance of papules (atrichia with papular lesions) and similar phenotypes appear when the key polyamine enzymes ornithine decarboxylase (ODC) and spermidine/spermine *N*¹-acetyltransferase (SSAT) are overexpressed. Both ODC and SSAT transgenic mice have elevated epidermal levels of putrescine, leading us to investigate the mechanistic link between putrescine and HR. We show here that HR and putrescine form a negative regulatory network, since epidermal ODC expression is elevated when HR is decreased and *vice versa*. We also show that regulation of ODC by HR is dependent on the MYC superfamily of proteins, in particular *MYC*, *MXI1* and *MXD3*. Furthermore, we found that elevated levels of putrescine lead to decreased HR expression but that the SSAT-TG phenotype is distinct from that of HR mutants. Transcriptional microarray analysis of putrescine-treated primary human keratinocytes demonstrated differential regulation of genes involved in protein-protein interactions, nucleotide binding, and transcription factor activity, suggesting that the putrescine-HR negative regulatory loop may have a large impact on epidermal homeostasis and hair follicle cycling.

Keywords

Polyamines; Hair cycle; Mouse models; Hairless; Gene regulation

INTRODUCTION

Previous studies by our lab and others have shown that the underlying cause of hairlessness in several different mouse strains is a mutation in the *hairless (HR)* gene (1-3). In humans, perturbation of *HR* expression results in two phenotypes: when *HR* is absent the result is

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CONFLICT OF INTEREST

The authors state no conflict of interest.

atrichia with papular lesions (APL; OMIM 209500; (4, 5) and when *HR* is overexpressed the result is Marie Unna hereditary hypotrichosis 1 (MUHH1; OMIM 146550; (6, 7).

Expression studies using mouse models have shown *that HR* is highly expressed throughout the hair follicle during the catagen stage of apoptosis and regression, therefore when *HR* is dramatically reduced, as is the case of the *HR* mutant mouse models, apoptosis becomes uncoordinated and the hair follicle ceases to cycle properly (8, 9). This results in the degenerated hair follicles and the formation of epidermal cysts that eventually take on the appearance of papules, hence the name of the disorder in humans, atrichia with papular lesions.

Epithelial carcinogenesis research has long utilized mouse lines that lack a hair coat, since this facilitates the application of carcinogens and ease of carcinoma monitoring (10-12). However, in addition to hair loss and papular lesions, mouse lines that have nearly absent levels of *HR*, such as *SKH-1* and *Rhino*, also exhibit an increased susceptibility to carcinogenesis. Recent work in our lab has shown that the underlying mechanism of this increased susceptibility to carcinogenesis is dysregulation of the $\text{NF}\kappa\text{B}$ pathway (13). $\text{NF}\kappa\text{B}$ dysregulation and hair loss occurs irrespective of the underlying molecular lesion in *HR*, since *SKH-1* and *Rhino* contain two distinct *HR* mutations. *SKH-1* animals have a proviral insertion into exon 6 of *HR* in manner similar to the *HRS/J* line (1), whereas the *Rhino* line contains a point mutation that results in a premature stop codon (2, 3). This allelic heterogeneity is also seen in humans, as there are many different recorded loss of function *HR* mutations and all of them result in the typical APL phenotype (14).

We previously postulated that the polyamine regulatory enzyme ornithine decarboxylase (ODC) and *HR*, despite having different roles in the hair follicle, may effect similar pathways, since the phenotype of mice overexpressing ODC under the K6 promoter (K6-ODC) is strikingly similar to *HR* mutants (15). Similar to animals lacking *HR* or upregulating ODC, SSAT under an endogenous promoter (SSAT-TG) also results in diffuse hair loss and the appearance of papular lesions (reviewed in (16). However, the molecular mechanisms underlying these apparent phenotypic similarities have not been explored.

It is well-established that polyamine metabolism plays a crucial role in almost every cellular function, including proliferation and differentiation, and it is controlled in large part by two key enzymes, ODC and spermidine/spermine N^1 -acetyltransferase (SSAT) (reviewed in (17)). ODC catalyzes the production of the lower order polyamine putrescine from L-ornithine. Putrescine is then used to synthesize the higher order polyamines spermine and spermidine by specific synthases. SSAT catalyzes the reverse by breaking down spermine and spermidine into putrescine (reviewed in (18)). Therefore, the net result of overexpressing either ODC or SSAT is the accumulation of the metabolite putrescine (Figure 1a).

The importance of controlled regulation of putrescine levels has been shown to be critical for epidermal homeostasis and hair cycling (19-21). It is noteworthy that both K6-ODC and SSAT-TG animals exhibit greatly increased levels of epidermal putrescine (22, 23). We therefore hypothesized that a mechanistic relationship exists between putrescine and *HR* that results in similar phenotypes in all three mouse lines.

Here, we aimed to define the mechanistic relationship between HR and the polyamine pathway and found that they form a negative regulatory loop, both *in vitro* and *in vivo*. We also go on to show that exogenous application of the polyamine putrescine leads to decreased *HR* expression, which is recapitulated in the SSAT-TG mice that have higher epidermal levels of putrescine and a distinct pattern of hair loss. Microarray analysis of putrescine treated primary human keratinocytes revealed that genes involved in protein-protein interactions, nucleotide binding, and transcription factor activity can be putrescine-regulated and thereby result in the distinct phenotype seen in SSAT-TG mice.

METHODS

Cell culture

Unless otherwise indicated, all cell culture supplies are from Life Technologies Grand Island, NY). Normal human keratinocytes (NHKs) from human foreskin and primary keratinocytes from an APL patient (24) were cultured on mitomycin C (2 µg/ml for 2 hrs, Sigma-Aldrich, St. Louis, MO) treated 3T3-J2 feeder layers (25, 26)

All transfections were performed at approximately 90% confluence using Lipofectamine 2000 (Life Technologies) and OptiMem following manufacturer's guidelines for 6-well plate transfections. 1 µg of either pCMV or a plasmid encoding either *HR* tagged with Flag or hODC (as indicated) were used in each transfection and all transfections were harvested for RNA extraction after 24 hrs. Cells were treated with either putrescine dihydrochloride (Sigma-Aldrich), α-difluormethylornithine (Sigma-Aldrich), or their respective carriers at approximately 90% confluence at the indicated concentrations. For RNA extraction, cells were washed twice with 1XPBS and harvested at the indicated time points into RLT Buffer (Qiagen, Valencia, CA), processed through a QiaShredder (Qiagen) and extracted with an RNeasy Mini Kit (Qiagen) following manufacturer's guidelines. RNA quantity was assessed on a Nanodrop 2000 (ThermoFisher, Wilmington, DE).

Quantitative RT-PCR

500 ng total RNA was used for the cDNA synthesis using Superscript III (Life Technologies) and a 2:1 ratio of random:oligo (dT) primers according to manufacturer's instructions.

Real time PCR was performed on an ABI 7300 (Life Technologies) using B2M as an internal control along with the Relative Quantification Assay and software. Primers (Life Technologies) were designed according to ABI guidelines and can be found In Supplemental Table 1.

All reactions were performed using ABI Power Sybr Master Mix, 200nM primers and 200ng cDNA. The data output was imported into the GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA), plotted, and the error calculated as standard error of the mean (SEM). Statistical analysis was performed as indicated using Student's T-test. For each experiment, n=4 replicates and *** p<0.0001, ** p<0.001, *p<0.05, error bars= mean +/- SEM.

Animals

SKH-1 mice were purchased from Charles River Laboratories (Kingston, NY). RhJ/LeJ (*rhino*), C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME), and SSAT-TG mice were kindly provided by Dr. Leena Alhonen, University of Eastern Finland. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Columbia University (IACUC Protocol Number: AC-AAAA5875).

Epidermal RNA extraction

Dorsal skin samples were taken from 10 week old SSAT-TG animals and WT littermates (n=4). The epidermis was separated from the dermis and lysed in RLT Buffer, processed through a QiaShredder, and RNA was extracted using an RNeasy Mini Kit following the manufacturer's instructions. Total RNA quantity was assessed on a Nanodrop 2000.

Hair cycle analysis

An approximately 2 cm² patch of dorsal fur was plucked from 25 day old SSAT-TG and WT littermates (n=4). The animals were left for 14 days until the WT animals showed hair regrowth following the timeline established by (27). For brightfield microscopy, pelage hair was plucked from 44 day old SSAT-TG and WT littermates (n=5) and preserved in Vectashield (Vector Labs, Burlingame, CA). Hair shafts were visualized using the 20X objective on a HRC Axiocam fitted onto an Axioplan2 fluorescent microscope (Carl Zeiss, Thornwood, NY) (28).

Microarray analysis

NHKs were treated with 1mM Putrescine for 24 hours and harvested for RNA extraction as described above (n=3). Total RNA was DNase treated and 90 µg of RNA was processed through a GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA). 15 µg of aRNA was hybridized onto a GeneChip Human Genome U133 Plus 2.0 Array. The resulting expression data was analyzed via GeneSpring GX (Agilent Technologies, Santa Clara, CA) and filtered on expression and volcano plot (p<0.05 and fold-change>1.5). GO terms were determined by DAVID software (29-31).

RESULTS

HR transcriptionally regulates *ODC* in human keratinocytes

We first assessed the level of ODC protein in primary normal human keratinocytes (NHKs) that were transfected with either an empty plasmid containing only the pCMV promoter or a plasmid encoding FLAG tagged HR under a pCMV promoter. As expected, the introduction of a FLAG-*HR* plasmid increased the amount of *HR* mRNA by approximately 68.50 times the amount found in pCMV transfected NHKs. Increased *HR* expression was accompanied by a concomitant decrease in *ODC* expression by approximately 53% (Figure 1a).

We also examined ODC expression the converse situation in which HR expression is significantly decreased due to a naturally occurring 22 bp deletion in exon (hereafter

referred to as HR keratinocytes) (26). As expected from the overexpression of HR, ODC expression in HR keratinocytes is significantly upregulated by approximately 2.5 times as compared to NHKs (Figure 1b). Thus, we decided to further explore how HR regulates ODC.

HR regulates ODC expression through the MYC related proteins *MXI1* AND *MXD3*

The *ODC* promoter is known to contain several MYC/MAX binding sites, known as E-boxes, and previous work has shown that *ODC* transcription can be regulated via direct binding of the MYC superfamily of proteins to these sites (32-34). While the MYC/MAX heterodimer can upregulate gene expression through direct binding to E-boxes, the MXD/MXI proteins are structurally similar to MYC and can repress gene expression through competitive binding of MAX and E-boxes (35). MXD1, MXI1, MXD3, and MXD4 are expressed to various degrees in the skin at different stages of epidermal development and differentiation (36). MXI1 is of particular interest since exogenous overexpression has been shown to repress *ODC* promoter activity *in vitro* (37).

We overexpressed FLAG-tagged HR in NHKs and assessed endogenous *MXD1*, *MXI1*, *MXD3*, *MXD4*, and *MYC* expression through relative quantitative RT-PCR. HR overexpression did not effect *MXD1*, *MXD4* or *MYC*, whereas both *MXI1* and *MXD3* were significantly upregulated by approximately 1.5 when compared to pCMV vector transfected NHKs (Figure 2a). We conclude that HR transcriptional regulation of *ODC* may occur through the MYC superfamily including regulation of MXI1 and MXD3.

In order to further elucidate a possible regulatory loop between the polyamine pathway and HR we then overexpressed ODC under a pCMV promoter and then treated the cells with the well-characterized ODC inhibitor DFMO (5 mM for 6 hours (38)). As we hypothesized, under untreated conditions, overexpression of ODC which may increase the amount of endogenous putrescine in the cells resulted in an approximately 50% decrease in HR expression (Figure 2b). DFMO treatment on cells transfected with an empty vector showed a similar decrease, while HR expression was rescued by DFMO treatment in cells transfected with a plasmid encoding ODC. Since HR expression is normally repressed by ODC, DFMO treatment of ODC transfected cells rescues ODC induced HR repression and returns HR to untreated, untransfected levels. These results indicated that a negative feedback loop exists between HR and the polyamine pathway in keratinocytes and led us to further investigate this phenomena.

Putrescine regulates HR transcription both *in vitro* and *in vivo*

Both SSAT-TG and K6-ODC mice have previously been shown to have exceptionally high epidermal levels of the polyamine putrescine, while exhibiting early hair loss in a manner that appeared similar to *Hairless* mutant mice (22, 23, 39). Therefore, we postulated that putrescine plays a role in the negative regulation of HR. In order to assess the effect of putrescine on HR expression, we began by exogenously treating NHKs with either 0.5 mM, 1.0 mM, or 2.0 mM putrescine for 12 hrs (Figure 3a). When the relative expression of HR was quantified, we found that as little as 0.5 mM putrescine significantly decreased HR expression to approximately 71% of that observed in untreated NHKs. 1.0 mM and 2.0 mM

putrescine further decreased *HR* to approximately 28% and approximately 40%, respectively, as compared to untreated cells (Figure 3a).

In order to validate the effect of putrescine on *HR* expression *in vivo*, we examined the level of *HR* in the epidermis of SSAT-TG mice. As compared to age-matched WT littermates, SSAT-TG animals had significantly lower amounts of *HR* that were equivalent to approximately 21% of WT expression (Figure 3b). Together with the *in vitro* data, this supported our hypothesis that putrescine negatively regulates *HR*.

SSAT-TG animals exhibit a unique pattern of hair loss

Previous reports stated that SSAT-TG mice begin to lose their hair at 21 days of age in a manner similar to *HR* mutant animals (23, 40). We found that the SSAT-TG mouse phenotype indeed begins at around 21 days of age, however the pattern of hair loss is distinct from *HR* mutant mice. *HR* mutants have a well-characterized wave-like loss of hair that follows the onset and progression of catagen in a rostral-caudal manner (41, 42). In contrast, following an apparently normal morphogenesis and initial coat of hair, SSAT-TG animals begin to permanently lose their hair in a random, patchy pattern beginning at around 21 days of age. By 24 days of age, SSAT-TG mice have patches of balding that are especially evident on the ventral surface of the animal (Figure 4a). At approximately 7 weeks old, SSAT-TG mice have large variations in hair loss including some animals that are largely devoid of hair (Figure 4b, left panel) and others that have retained a large portion of their hair (Figure 4b, right panel).

Due to the patchy nature of the hair loss, we postulated that it may occur, in part, due to a hair cycling defect. We plucked pelage fur from WT and SSAT-TG mice at 25 days of age, when the animals were in telogen and monitored them for 14 days post-plucking (27). As expected, WT animals immediately entered anagen and regrew hair in the plucked area; however, SSAT-TG animals failed to regrow hair and maintained a denuded patch throughout the protocol (Figure 4c). This indicated that SSAT-TG mice have a failure during catagen that results in an inadequately anchored hair in a manner similar to the catagen defect described in *HR* mutant animals (9). The difference being that in the *HR* mutant mice, hair fibers are actively shed during catagen, while in the SSAT-TG mouse model the fibers are abnormally retained in the follicle and are only dislodged after mechanical stimulation.

We further investigated this hair loss by plucking pelage hairs from 44 days and visualizing them under brightfield microscopy. This showed that, as expected (28), the WT animals had well anchored hairs that retained a large amount of follicular material and created a club-like appearance (Figure 4d, top panels). The SSAT-TG hairs were strikingly abnormal and appeared dystrophic with very little follicular material attached (Figure 4d, bottom panels). These observations support the hypothesis that SSAT-TG mice have inadequately anchored hairs that are abnormally retained in the hair follicle and become easily dislodged which results in permanent hair loss.

Putrescine differentially regulates gene expression in keratinocytes

The unique pattern of hair loss exhibited by the SSAT-TG mice led us to postulate that HR was not the only pathway regulated by putrescine in keratinocytes. In order to interrogate other possibly affected pathways, we extracted RNA from NHKs treated with 1mM putrescine and analyzed gene expression using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Using GeneSpring software, and filtering on significantly different genes ($p < 0.05$ and fold-change > 1.5) a relatively small list featuring 15 genes was generated (Supplemental Table 2). As indicated on Supplemental Table 2, a selected number of the genes (7/15) were validated using relative quantity RT-PCR. DAVID software (29-31) was utilized to extract common molecular function gene ontology terms and the list was divided into two groups containing the majority of the genes (Supplemental Figure 1a). The largest group contained 11 genes that were identified as being involved in protein-protein interactions (*BAX*, *ADRA2C*, *UBAP2L*, *DAB2IP*, *EXTL3*, *ZBTB22*, *PDZD2*, *KLHL18*, and *ATF3*), while the second group was a set of 6 genes that exhibit nucleotide-binding ability (*IQCA1*, *KSRI1*, *ULK4*, and *ZNF215*). Of particular interest was a third group of genes that had multiple functions and overlapped the first two groups; it contained 4 genes identified as related to transcription factor activity (*ZBTB22*, *ATF3*, and *ZNF215*). Two genes (*LAD1* and *TRIM68*) did not functionally relate to the other genes. This data illustrates that putrescine is capable of modulating the activity of a selected group of protein activities via a small number of key genes.

DISCUSSION

We show here for the first time that the polyamine putrescine and HR form a negative regulatory loop that potentially plays a role in a variety of epidermal pathways, including hair follicle cycling and carcinogenesis. Our findings here provide evidence that the overexpression of HR induces upregulation of MXI1 and MXD3 with concomitant decreases in *ODC* expression. Unpublished data from our lab indicates that this upregulation of MXI1 and MXD3 by HR may be accomplished through epigenetic mechanisms (A.M. Christiano and L. Liu, 2013). From the available data we believe that HR acts as to demethylate H3K9 in the transcription regulatory elements (TRE) of MXI1, thereby increasing its transcription. Chromatin immunoprecipitation studies to assess the levels of H3K9 methylation in the TRE of MXI1 found partial H3K9 demethylation when HR is overexpressed. Concomitant with the demethylation was a significant increase in MXI1 expression indicating that transcription was directly impacted.

The data presented here shows that MXI1 regulates endogenous *ODC* transcription through the MAX/MYC binding sites in the *ODC* promoter region (Figure 2a). It should be noted that while MYC and MXI1 have previously been implicated in *ODC* promoter regulation in reporter assays (32, 37), to our knowledge, this is the first time that the MYC superfamily has been implicated in the regulation of endogenous *ODC*.

There is a feedback loop between HR and *ODC* as *ODC* is capable of negatively regulating HR expression. Such regulation can be seen when *ODC* is overexpressed or co-expressed with DFMO, however DFMO alone results in a somewhat anomalous downregulation of HR expression (Figure 2b). We hypothesize that there downstream of DFMO regulation of *ODC*

there are unknown consequences on HR expression, such as alternative regulatory pathways through spermidine and spermine. Clearly, more research focusing on polyamine regulation of HR remains to be done.

Previous studies have shown that the two transgenic mouse lines with increased epidermal putrescine levels, K6-ODC and SSAT-TG, share overt phenotypic similarities with the *HR* mutant mice lines (15, 40), leading us to postulate a mechanistic connection between putrescine and *HR* expression. We found that putrescine indeed regulates *HR* expression; *in vitro* with exogenous application on NHKs and through the induction of endogenously higher levels of ODC and *in vivo* in the SSAT-TG mice. However, examination of the pattern of hair loss in SSAT-TG mice revealed that it is distinct from *HR* mutant mice leading us to further speculate that *HR* is not the only gene regulated by putrescine.

Rather than the well-characterized wave-like pattern of hair loss, SSAT-TG mice begin to lose their hair in a patchy, random fashion during the first telogen. The random fashion and lack of follicular material attached to plucked hair shafts argues that, similar to the *HR* mutant phenotype, hair is lost due to a defect in catagen that results in an improperly anchored club hair (9). However, the *HR* mutant animals exhibit a coordinated loss of hair indicating an active process is at work while the SSAT-TG mutants abnormally retain their club hair fibers and only shed them after mechanical stimulation thereby accounting for the apparent random and individualized hair loss in the SSAT-TG population. *HR* mutations are primarily considered to lead to dysregulated apoptosis and proliferation (8, 26), and the downregulation of the *HR* undoubtedly accounts for the defective club hair formation seen in the SSAT-TG animals. However, we hypothesize that the lack of active hair shedding is due to one of the many other pathways that putrescine effects.

Our microarray analysis of putrescine treated keratinocytes showed that a variety of genes are differentially regulated, primarily those involved in protein-protein interactions, nucleotide binding, and transcription factor regulation. This data argues that putrescine may influence a wide variety of pathways even though a relatively small number of genes were identified as significantly changed with putrescine application.

The application of exogenous putrescine may influence gene expression through many different mechanisms including DNA conformation and RNA stability (43, 44). Previous research has shown that polyamines induce a conformational change of DNA from the B form to the Z form that is associated with increased transcriptional activity (45, 46). Additionally, polyamine depletion is thought to increase RNA stability and thereby modulate the half-life of RNA transcripts (44). Altogether this indicates that putrescine may mediate gene regulation in human keratinocytes through a multitude of ways.

We further postulate that putrescine may regulate gene expression, including *HR* expression, through an epigenetic mechanism since an increasing body of evidence points to a role for polyamines in epigenetics (47-49). Polyamine analogues have been found to inhibit lysine-specific demethylase 1 (LSD1), a key demethylase of the histone 3 lysine 4 (H3K4) activating mark (50). Inhibition of LSD1 and concomitant methylation of H3K4 results in the re-expression of silenced genes (51), and we therefore speculate that the *HR* and other

putrescine regulated genes may be LSD1 gene targets. It has also been found that *in vivo* overexpression of ODC results in elevated activation of HDAC and HAT (47, 48). Alterations in histone acetylation provide a further mechanism through which ODC and SSAT expression can affect gene transcription, however further work is necessary to elucidate the connection between polyamines and epigenetic gene regulation in the hair follicle.

Our data provide the first evidence for a negative regulatory loop between HR and the putrescine-generating enzymes SSAT and ODC (Supplemental Figure 1b). Putrescine has previously been thought of as a “building block” for the higher order polyamines, and therefore research has not focused exclusively on a cellular effect for putrescine. However, our findings show that putrescine plays a key role in a negative feedback loop that has wide ranging consequences in the epidermis and hair follicle. Putrescine-mediated gene regulation clearly maintains a delicate balance of gene expression in the epidermis and hair follicle, and could be responsible for a variety of functions including hair cycling and tumorigenesis due to the dynamic nature of polyamine expression (52). Future studies may uncover additional regulatory roles for this previously understudied polyamine in the skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HR regulates the polyamine pathway through negative regulation of ODC

- a) NHKs were transfected with either an empty or a plasmid encoding FLAG tagged *HR*. The relative quantities of *HR* and *ODC* mRNA were assessed via RT-PCR. When keratinocytes were transfected with FLAG-*HR*, *HR* expression significantly increased while endogenous *ODC* significantly decreased.
- b) The relative quantity of ODC was assessed via RT-PCR in passage matched NHKs and HR keratinocytes lacking functional HR. HR keratinocytes showed significantly increased amounts of ODC as compared to the amount found in NHKS.

Figure 2. HR regulates ODC expression via *MXII* and *MXD3*

a) NHKs were transfected with either an empty plasmid containing only a pCMV promoter or a plasmid encoding FLAG tagged *HR* under the control of a pCMV promoter. When *HR* was overexpressed in keratinocytes, the levels of *MXII* and *MXD3* significantly increased.

b) NHKs were transfected with either an empty plasmid containing only a pCMV promoter or a plasmid encoding *ODC* under the control of a pCMV promoter. After 24 hours, cells were treated with either PBS or DFMO. When *ODC* is overexpressed, *HR* expression is lowered, an effect that is abolished with DFMO application.

Figure 3. Putrescine downregulates *HR* transcription both *in vitro* and *in vivo*

a) NHKs were either left untreated or treated with 0.5 mM, 1.0 mM, or 2.0 mM of putrescine for 24 hours. The extracted RNA was used for relative quantity RT-PCR, which showed that at all putrescine concentrations *HR* expression, was significantly decreased *in vitro*.

b) SSAT-TG and WT littermates were sacrificed at 10 weeks of age and epidermal RNA extracts were prepared from each animal. Relative quantitative RT-PCR showed that when *SSAT* is overexpressed *in vivo*, epidermal levels of *HR* are significantly decreased.

Figure 4. SSAT-TG mice exhibit patchy hair loss

- a) 24 days old SSAT-TG mice begin to lose hair in a random, patchy fashion.
- b) By 10 weeks old, the SSAT-TG mice are largely hairless, however, some animals retain more hair than others.
- c) When hair is plucked from SSAT-TG and WT at 25 days old, anagen is induced in the WT animals and the hair regrows. In contrast, plucked SSAT-TG animals never regrow hair.
- d) When pelage hair from 44 day old animals is plucked and visualized under brightfield, a clear difference between WT and SSAT hair shafts can be seen. WT hairs (upper panels) retain a large amount of follicular matter and appear more club-like, whereas SSAT-TG hairs (lower panel) have little follicular material attached.