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A new tool for conditional gene manipulation in a subset of keratin-expressing epithelia

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Abstract

Megsin is a Serine protease inhibitor (Serpin) that has known expression in kidney mesangial cells. Here, we report the generation and characterization of a bacterial artificial chromosome (BAC) transgene expressing *Cre* under the control of *Megsin* regulatory elements. When crossed to the *ROSA26R-lacZ* reporter mice, the *Megsin-Cre* transgene mediates *loxP* recombination primarily in the skin, forestomach and esophagus, but surprisingly not in the mesangial cells. Within the skin, cells in all epidermal layers and the hair follicle cells expressed *Cre*. This transgene also has uniform expression in the epithelium of the forestomach and esophagus. Conditional deletion of *Adam10*, a gene known to have important functions in skin development, by using this *Megsin-Cre* transgene led to severe skin defects. In addition, these mutants appear to have reduced folds and surface area in the forestomach. These results show that the *Megsin-Cre* transgene can mediate *loxP*-recombination in all epidermal layers of the skin, the hair follicle cells, as well as in the epithelium of the forestomach and esophagus, all of which have known expression of various keratins. This *Megsin-Cre* transgene can serve as a new tool for conditional genetic manipulation to study development and diseases in the skin and the upper digestive tract.

Keywords

Megsin; *Adam10*; epidermis; forestomach; esophagus

Introduction

Mesangial cells play critical roles in maintaining the structure and function of the glomerulus in the kidney (Herrera, 2006). Injury of mesangial cells can lead to various glomerular diseases with marked upregulation of Megsin (Miyata *et al.*, 1998). *Megsin*, a member of the Serpin (serine protease inhibitor) superfamily, is highly conserved among different species (Inagi *et al.*, 2003). It is expressed in mesangial cells and reportedly plays a role in Mesangial cell function and homeostasis (Miyata *et al.*, 1998). Recent studies identified upregulation of *Megsin* in many human kidney diseases and the polymorphisms of Megsin gene are associated with the susceptibility and/or progression of kidney disease (Li *et al.*, 2004; Miyata *et al.*, 2007; Xia *et al.*, 2006a; Xia *et al.*, 2006b). There are several Cre lines showed reporter activity in mesangial cells, but the Cre expression directed by these transgenes is widespread, limiting their use as a cell type-specific driver in studies targeting

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mesangial cells (Cuttler *et al.*, 2011; Humphreys *et al.*, 2010). Considering the important roles of Mesangial cells in kidney development and chronic diseases, the need for generating a mesangial cell-specific Cre transgenic line is urgent. An earlier attempt using a segment of the *Megsin* promoter to generate a Cre line was unsuccessful due to the absence of Cre expression (Gawlik and Quaggin, 2004; Kohan, 2008; Miyata *et al.*, 1998). We thought to change the approach by using BAC transgenes in our efforts to increase the probability of having strong and faithful expression of Cre in the mesangial cells.

The ADAMs (A Disintegrin and Metalloproteinase) are a family of peptidase proteins. They are transmembrane proteins belong to the zinc protease superfamily and contain a disintegrin and metalloprotease domain. The ADAMs have been implicated in a wide range of biological processes from the control of membrane fusion, cell fate determination, to pathologic processes such as cancer and inflammation (Seals and Courtneidge, 2003). *Adam*10 is capable of proteolytic cleavage of a wide range of substrates, including type IV collagen, EGF, Ephrin, chemokines, prion precursor protein, and many others (Klein and Bischoff, 2011). Most importantly, Adam10 mediates the S2 cleavage of the Notch ligands, effectively serving as an upstream activator of the Notch signaling pathway (Hartmann *et al.*, 2002; Jorissen *et al.*, 2010; Weber *et al.*, 2011). Recent studies in transgenic models have shown that *Adam10* is involved in the regulation of skin morphogenesis and homeostasis. Epidermal deletion of *Adam10* during skin morphogenesis led to a precocious epidermal differentiation and hyperproliferation in adult epidermis (Weber *et al.*, 2011). Conversely, overexpression or increased activation of *Adam10* in human also led to chronic skin disease (Dumortier *et al.*, 2010; Maretzky *et al.*, 2008).

Conditional gene targeting using the *Cre*/*loxP*-mediated recombination system offers a powerful approach to manipulate gene function under the control of a tissue/cell specific promoter in a spatially and temporally specific manner. Here we generated a bacterial artificial chromosome (BAC) transgene expressing *Cre* under the control of *Megsin* regulatory elements. The *Megsin-Cre* transgene mediates *loxP* recombination occurred primarily in the skin, forestomach, and esophagus, but surprisingly not in the mesangial cells. Conditional deletion of *Adam10*, led to severe skin defects and forestomach epithelium anomalies. The *Megsin-Cre* transgene mainly mediate *loxP*-recombination in tissues that have known expression of various keratins. It can serve as a new tool for conditional genetic manipulation to study development and diseases in these tissues.

Results and Discussion

To provide a new genetic tool to investigate the development and pathology of the glomerular and kidney diseases, we set out to make a transgene that will direct *Cre* expression specifically under the promoter of the gene *Megsin*. Since BAC (Bacterial artificial chromosome) transgenes have higher capacity to carry most, if not all, of the regulatory sequences necessary to recapitulate the expression pattern of the endogenous gene, we set out to construct the *Cre* transgene in a BAC clone covering the *Megsin* gene but not any other full length known genes. We used recombineering (recombination-mediated genetic engineering) to replace the first coding exon of *Megsin* with the coding sequence of an I-*Cre* (codon **I**mproved ***Cre***) fused to a nuclear localization signal (NLS) (Fig. 1). Thus, *Cre* expression is effectively under the control of the regulatory elements of the *Megsin* gene. This cassette destroys the coding sequence of *Megsin* and terminates the transcription (by the transcription termination in the *Cre* cassette) upstream of exon 3 of the *Megsin* gene.

After pronuclei injection, one healthy and fertile founder was produced in a *C57Bl/6xCBA* hybrid background. To test the efficiency and the specificity of the transgene expression, we

crossed the founder and its offspring to the *ROSA26R-lacZ* reporter mice (Soriano, 1999). To our surprise, *Megsin-Cre*-mediated *loxP* recombination was not detected in the mesangial cells, revealed by histochemical detection of β-galactosidase activity (Data not shown). We are not certain about the reason for the lack of mesangial expression. Mutations in *cis*-acting element important for mesangial expression or the suppression of such elements by the local chromosomal environment are among the possible explanations.

Although no expression in the mesangial cells, we found strong β-galactosidase activities in epidermis and hair follicles of both ventral and dorsal skin at E14.5 (Fig. 2A) and postnatally (Fig. 2B and C). The skin consists of several layers, the stratum corneum, the epithelial layer (epidermis), the connective tissue layer (dermis) and the adipose layer (hypodermis) underneath (Takahashi *et al.*, 1998). The epidermis is a stratified squamous epithelium that contains several keratinocyte layers, including the granular layer, spinous layer and basal layer. Keratinocyte maturation is a process that relatively undifferentiated keratinocyte progenitors in the basal layer differentiate, in sequential order, into intermediate spinous layer, granular layer, and terminally differentiated corneocytes in the cornified layer. These layers can be identified by individual keratin markers. For example, *Keratin10* (*K10*) is expressed in the suprabasal layer of the epidermis and *Keratin 14* (*K14*) is expressed in the basal layer of epidermis (Koch and Roop, 2004; Margadant *et al.*). To study the expression of the *Megsin-Cre* transgene in the skin at the cellular level, we used K10, K14 and E-cadherin (E-Cad) to analyze skin sections from *Megsin-Cre*/+; $ROSA26R^{lacZ}$ mice and *Megsin-Cre*/+; $ROSA26R^{YFP}$ mice, respectively. The *Megsin-Cre* transgene was expressed in all layers of the epidermis, as indicated by colocalization of the *YFP* with the epithelial marker E-cadherin (Fig. 2D–F), with the suprabasal layer marker K10 (Fig. 2G–I), and with the basal layer marker K14 (Fig. 2J–L). Expression in the hair follicle epithelium was also noted (Fig. 2B–L).

Besides skin expression, strong expression of the transgene was also detected in the mouse forestomach (Fig. 3A–I) and esophagus (Fig. 3J–O). Mouse forestomach is composed of a stratified squamous epithelium with high levels of similarity to the esophageal epithelium (Takahashi *et al.*, 1998). The development of adult mouse stomach epithelium begins with the initiation of endoderm cytodifferentiation in late fetal life. E12.5 mouse gastric mucosa is lined with a simple undifferentiated epithelial monolayer that shows a pseudostratified appearance by E14.5. The keratinized stratified squamous forestomach epithelium starts to differentiate by E16.5 and organizes into primordial buds by E18, (Karam *et al.*, 1997; Spencer-Dene *et al.*, 2006). *Megsin-Cre*-mediated *loxP* recombination occurred in the epithelium of forestomach, but not in the glandular hindstomach, as shown by *YFP* signal and X-gal staining in Fig. 3A–C. To further determine the specific cell types where *Megsin-Cre* was expressed in the forestomach, we examined the transgene expression together with epithelial and smooth muscle markers, E-cadherin and SMA, respectively. We found that the *Megsin-Cre*-mediated *loxP* recombination only occurred in the epithelium, but not the muscle layers of the forestomach as revealed by the colocalization of *YFP* signal and E-cadherin staining (Fig. 3D–F), as well as the mutual exclusion of the *YFP* and SMA signals (Fig. 3G–I). Similar expression pattern was also found in esophagus epithelium, indicated by the colocalization of *YFP* signal and E-cadherin (Fig. 3J–L) and the mutual exclusion of *YFP* and SMA signals (Fig. 3M–O). The expression of Cre in the skin and forestomach may be driven by the Megsin transcriptional regulatory elements or influenced by regulatory sequences that are unrelated to Megsin but near the transgene insertion site. Reports of *Megsin* transcripts in the skin and the forestomach appear to suggest that expression in these tissues is related to *Megsin* promoter activity (Toulza *et al.*, 2007).

In order to show the *Megsin-Cre* transgenic can efficiently and specifically mediate *loxP* recombination for gene function studies, we have selectively crossed the *Megsin-Cre* mice

with the mice carrying a floxed allele of *Adam10*. Previous study of *Adam10* deletion using a similar transgenic *Cre* line, *Keratin 14-Cre* (*K14-Cre*), has demonstrated *Adam10* as a central regulator of skin development and maintenance (Weber *et al.*, 2011). The *K14* promoter drives *Cre* expression specifically in the basal layer of the epidermis and its appendages, such as hair follicles and sebaceous glands (Hafner *et al.*, 2004). The *K14-Cre* driven epidermal *Adam10* deletion led to perinatal lethality due to a perturbed water barrier function. The mutants also had a strong reduction in epidermal thickness and absence of sebaceous glands (Weber *et al.*, 2011). The *Megsin-Cre* mouse has hair follicle expression early (E14.5). In postnatal mice, *Cre* activity is detected in all epidermal layers of the skin and in the hair follicles in the dermis. Unlike the phenotypes shown in *K14-Cre; Adam10* mutant mice, the *Megsin-Cre; Adam10* mutant mice were able to survive into adulthood, with progressive hair loss. Hair loss started out in patchy areas at the midline of both dorsal and ventral skin, but progressed to more severe and broader hair loss throughout the body (Fig. 4A–C). The dynamic pattern of hair loss in these mutants may be caused by the combined effects of *Adam10* deletion and the gradient anagen spreading waves of hair follicles (Plikus and Chuong, 2008). By H&E staining we did not detect any obvious reduction in the thickness of the epidermis or the absence of sebaceous glands, but we found the presence of keratin cysts in the dermis which may contribute to the destruction of hair follicles and the eventually hair loss (Fig. 4E). Keratin cyst formation is often interpreted as an adoption of an epidermal differentiation program. Adam10 serves as a key regulator of Notch signaling pathway that plays important roles in epidermal differentiation. The disruption of Notch signaling by *Adam10* deletion in the developing hair follicle may lead to perturbation of terminal differentiation program, causing the diversion of Notch-deficient hair follicles to epidermal cysts (Demehri and Kopan, 2009; Vauclair *et al.*, 2005; Weber *et al.*, 2011). The differences in the phenotypes of the *K14-Cre; Adam10* and the *Megsin-Cre; Adam10* mutant mice appear to reflect the temporal-spatial differences of *Cre* activities from these two transgenes.

Because of the finding of *Megsin-Cre* expression in the stomach and esophagus, we also examined the *Megsin-Cre-Adam10* control and mutant mice for potential defects in these structures. None of the *Megsin-Cre-Adam10* mutant mice showed overt digestive problems. All mutants grew at similar rates when compared to their littermates, as determined by body weight measured at regular intervals. Histological analyses showed no significant differences in esophagus and glandular hindstomach between the controls and the mutants (Fig. 4F-G, J-K). However, *Megsin-Cre induced Adam10* deletion resulted in hypoplasia in the forestomach (Fig. 4H-I). The forestomach of these mutant mice showed decreased depth of gastric pits in the forestomach (mutants 62.5 ± 22.8 μm; controls 173.4 ± 54.2 μm; $p<0.001$) (Fig. 4I). These structural changes, however, were not sufficient to cause detectable digestive phenotypes in the mutants under normal diet and housing conditions. It remains to be investigated if these mutants are more susceptible to develop digestive problems when fed different diet regimens (for example, high-fiber or high-fat diets).

There are a number of transgenic mouse lines that can drive Cre expression in the skin (Schneider, 2012). Among them, the *Krt1-15-CrePR1* transgene is very different from the *Megsin-Cre* since it used the *Keratin 15* promoter to target the hair follicle bulge stem cells in an inducible manner (Morris *et al.*, 2004). Two other Cre lines, *K5-Cre* and *K14-Cre* drive Cre expression in a similar group of cells that *Megsin-Cre* targets (Hafner *et al.*, 2004; Ramirez *et al.*, 2004), However, minor differences in the dynamics of Cre expression may lead to functional consequences, as evidenced by the phenotypic difference seen between the *K14-Cre; Adam10* mutants and the *Megsin-Cre; Adam10* mutants. In addition, these three lines seem to have very different expression pattern outside of skin. In particular, both *K5-Cre* and *K14-Cre* have expression in the oocytes, leading to ubiquitous Cre-mediated *loxP* recombination when the mother carries the Cre transgene. When the father carries the Cre

transgene, Cre activities can be found in tongue, thymic epithelium, and other places (Hafner *et al.*, 2004; Huang *et al.*, 2009; Ramirez *et al.*, 2004). These differences make each of these Cre lines with skin expression unique tools for genetic studies.

Taken together, we generated a new transgenic mouse line, *Megsin-Cre*, which exhibits *Cre* activity specifically in epithelial cells of the skin epidermis, hair follicles, forestomach, and esophagus. Conceivably, in addition to the gene manipulations similar to the *Adam10* study presented here, this transgene can also be used for lineage tracing studies in normal development and in diseases states.

Methods

Generation of the BAC transgene construct by Recombineering

A BAC clone carrying the murine *Megsin* gene was purchased from Invitrogene (Clone # RP23-174I22). This BAC clone (*Megsin*BAC), carrying a chloramphenicol (Cm) resistant cassette, was transformed into *DH10B* host cells. We then introduced another plasmid *pRedET*, carrying a tetracycline (Tet) resistant cassette, into these *DH10B* cells that already have *Megsin*BAC. The presence of the *pRedET* plasmid restores the ability for selected types of recombination in the *DH10B* cells. At the same time, we used PCR to introduce 2 homology arms to the plasmid *phCre2.myc.nuc.FRTN1.amp.FRT* that expresses a codon-improved *Cre* with a small *Myc* tag and nuclear localization sequence (NLS) (a gift from Dr. Günther Schütz) (Casanova *et al.*, 2001). The *Frt.Amp.Frt* cassette provides ampicillinresistance (Amp) for the intermediate cloning steps and was later removed before the construct was finalized. The homology arms were designed for replacing a stretch of the *Megsin* gene with the *Cre* cassette by recombineering. The left homology arm consists of the 50 nucleotides directly 5′ of the ATG start codon of *Megsin*. The right homology arm consists of a 50 nucleotides long sequence in intron 2 of *Megsin*. In this design, after recombineering, the coding sequence (CDS) in the first coding exon (exon 2) of *Megsin* would be replaced by the *Cre*-expressing cassette. Therefore, the expression of *Cre* would be subjected to the control of the *Megsin* promoter. The multiple transcriptional stop signals would ensure that the partial *Megsin* gene left on the BAC clone is not transcribed. The purified PCR product (*Megsin.left.arm_phCre2.myc.nuc.FRTN1.amp.FRT_Megsin.right.arm*) was electroporated into the competent *DH10B* cells that already had both *MegsinBac* and *pRedET*. The electroporated cells were selected on plates with Cm, Tet, Amp, and Arabinose (for inducing the required recombinases from the *pRedET* plasmid). The *Megsin*.left.arm_ph*Cre*.myc.nuc.FRT.AMP.FRT_*Megsin*.right.arm was a PCR product and could not be reproduced by the bacteria without recombination that incorporates it into the BAC. Thus, the Amp resistant clones were screened for the ones where recombination occurred between the homology arms of the PCR product and the corresponding *Megsin* sequence on the BAC. The *Frt-Amp-Frt* cassette was subsequently removed by the introduction of a *p706FLPE* plasmid (a gift from Dr. Stewart) (Zhang *et al.*, 1998). Every cloning step was confirmed by restriction digestion, PCR, and/or sequencing. The final transgene construct (*Megsin-Cre*/BAC) was subjected to sequencing to ensure that everything proceeded as planned and no unwanted mutation was introduced.

Generation of the transgenic mice

All animal studies have been approved by IACUC (Institutional Animal Care and Use Committee) at Washington University School of Medicine. The transgene construct was purified by using the Qiagen large DNA Construct kit and was dialyzed by using the transgene injection buffer (10mM TRIS, pH7.4, 0.1mM EDTA). The construct was injected into the pronuclei of fertilized oocytes from *C57Bl/6xCBA* hybrids. The presence of the

transgene in the founders and their offspring was detected by PCR using primers MCF 5′ CCATCCAACAGCACCTGGGCCAGCTCAACA 3′ and MCR 5′ CCACCATCGGTGCGGGAGATGTCCTTCACT 3′. The *ROSA26R-lacZ* reporter mice were described previously (Soriano, 1999) and were genotyped by using primers WS268 5′ GTTATCAGTAAGGGAGCTGCAGTGG 3″ WS270 5″ AAGACCGCGAAGAGTTTGTCCTC 3′ and WS271 5″ GGCGGATCACAAGCAATAATAACC 3′ to amplify a wild-type band of 500 bp and a band of 250 bp corresponding to the *ROSA26R-lacZ* allele. PCR conditions were: 95°C, 2′, 35 × (94°C 30″; 59.5°C, 30″ 72°C, 30″), 72°C, 5′. *ROSA26R-LacZ* mice were purchased from the Jackson Laboratories (Bar Harbor, ME). *YFP* mice were kindly provided by Dr. Frank Costantini (Srinivas *et al.*, 2001).

Histology and Immunohistochemistry

10 μm cryostat sections of embryos or tissues were collected. 5-Bromo-4-chloro-3-indolyl-D-galactoside (Xgal) staining on cryostat sections was performed as described (Chang *et al.*, 2004). Immunostaining on cryostat sections was performed as previously described (McDill *et al.*, 2006). Antibodies used were: anti-α-SMA antibody (Sigma, St. Louis, MO, 1:500), anti-Keratin10 antibody (Covence, Princeton, NJ, 1: 1000), anti-Keratin 14 antibody (Covence, Princeton, NJ, 1: 1000), anti-E-cadherin antibody (Abcam, Cambridge, MA, 1:200). Appropriate fluorescent conjugated secondary antibodies (Molecular Probes, Invitrogen, Carlsbad, CA, 1:1000) were used to detect the corresponding primary antibodies.

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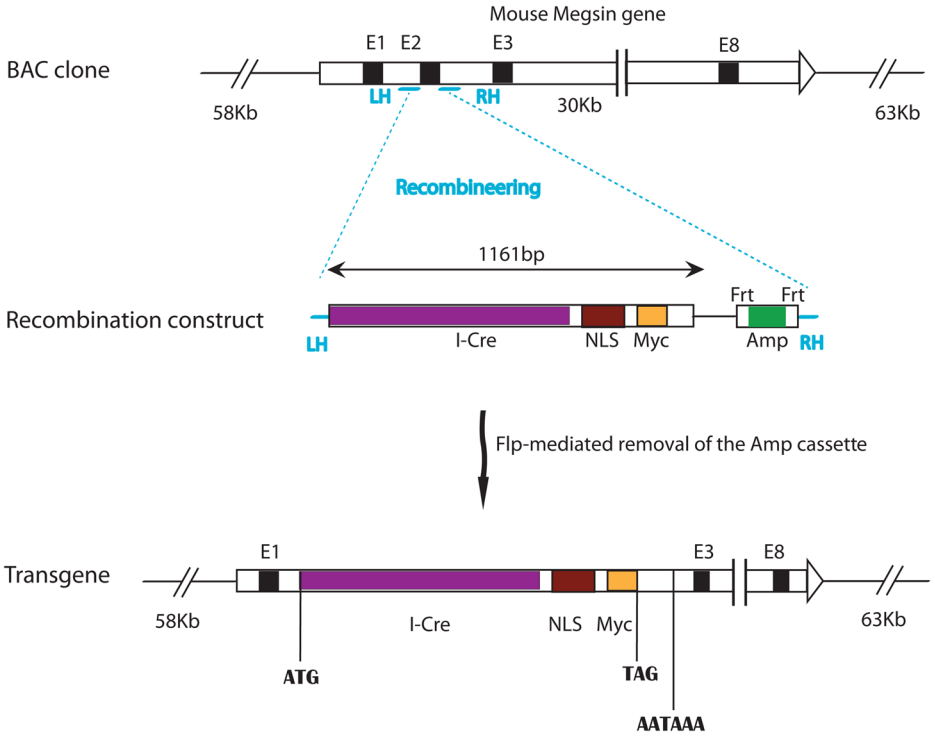

Figure 1. Generation of a Megsin-Cre transgene using recombineering in a BAC clone

The entire *Megsin* gene resides in the middle portion of the BAC clone we used to build the *Cre* transgene. No other complete genes are present in this BAC clone. The recombination construct has two homology arms (LH: left homology; RH: right homology) that are homologous to the sequences immediately 5′ and immediately 3′ to exon 2 of *Megsin*, respectively. The recombination construct also has the coding sequence for a codon-improved *Cre* (I-*Cre*), a nuclear localization signal (NLS), and a Myc tag. In addition, it has an *Frt*-flanked ampicillin resistance gene cassette for selection. Exon 2 (E2) of *Megsin* in the BAC clone was replaced by the recombination construct between the two homologyarms after recombineering. An additional round of transient Flp expression eliminates the Amp cassette to avoid bringing unnecessary prokaryotic sequences into the mammalian genome.

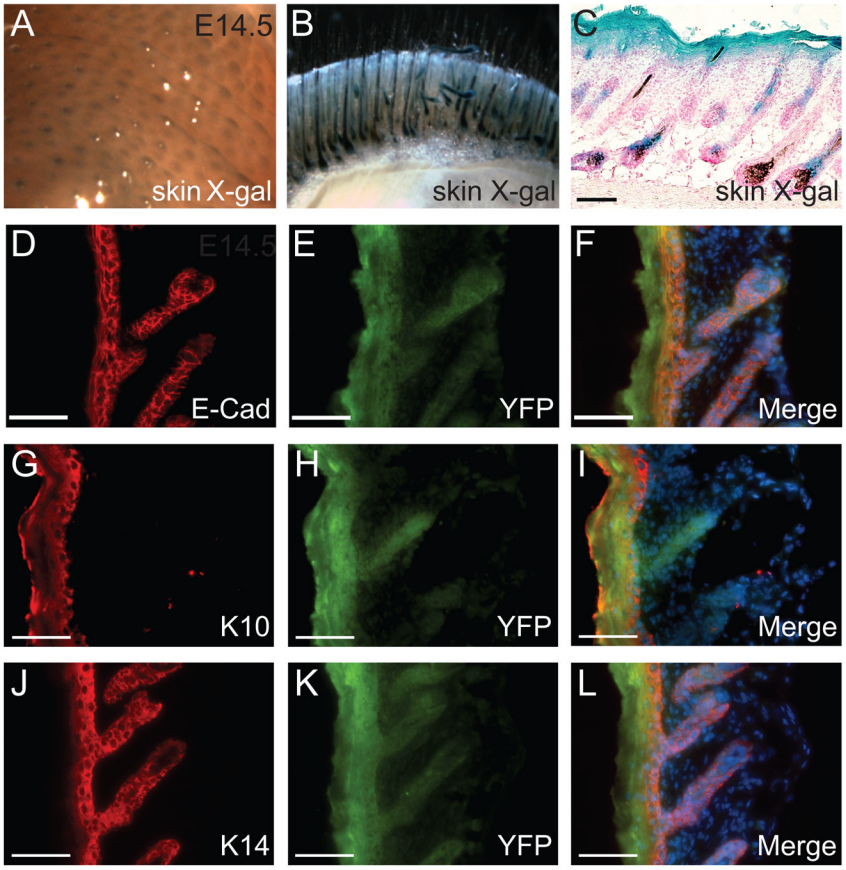

Figure 2. The Megsin-Cre transgene has expression specifically in the skin

Whole-mount β-galactosidase assay (A–B) and X-gal analysis on skin cryostat section (C) revealed the occurrence of *Cre*-mediated *loxP* recombination in the hair follicles and epidermis. A: E14.5 embryos. B: skin of P3 transgenic mouse. C: skin section from P3 transgenic mouse. Immunohistochemical analysis indicated *Megsin* was expressed in all epidermal layers (D-L). D-F: *YFP* colocalized with E-cadherin staining in the skin epithelium, G-I: *YFP* colocalized with K10 staining in the suprabasal layers of the epidermis and other cornified stratified epithelia, J-L: *YFP* colocalized with K14 staining in the basal layer of the epidermis. Scale bar: 100 μm.

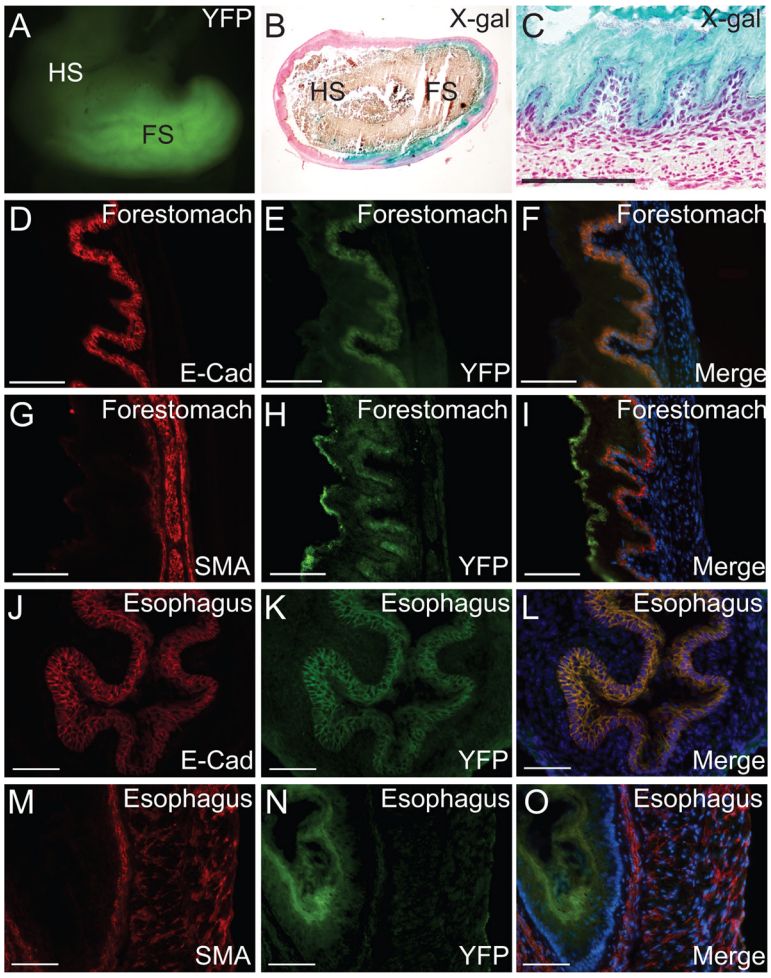

Figure 3. Megsin-Cre expression is in the epithelium of the forestomach and esophagus

Transgenic expression of *Megsin-Cre* in whole mouse stomach was detected in the forestomach indicated by *YFP* signals in whole-mount preparation from a *Megsin-Cre*/+, *ROSA26R*$^{YFP/+}$ mouse (A) and β-galactosidase activity assays on sections from a *Megsin-Cre*/+, *ROSA26R*$^{lacZ/+}$ mouse (B–C). It was undetectable in the glandular hindstomach. Expression of *Megsin-Cre* in the forestomach of transgenic mice (D–I). As indicated by the epithelial marker E-cadherin and the smooth muscle marker αSMA, *Megsin-Cre* expression was restricted to the epithelium of the forestomach (D–F) and no expression was seen in the smooth muscle layers (G–I). Colocalization of *YFP* and E-cadherin and mutual exclusion of *YFP* and α-SMA indicated the specific expression of *Megsin-Cre* in the esophagus epithelium of transgenic mice (Fig. 3J–O). FS: forestomach, HS: glandular hindstomach. Scale bar: 100 μm.

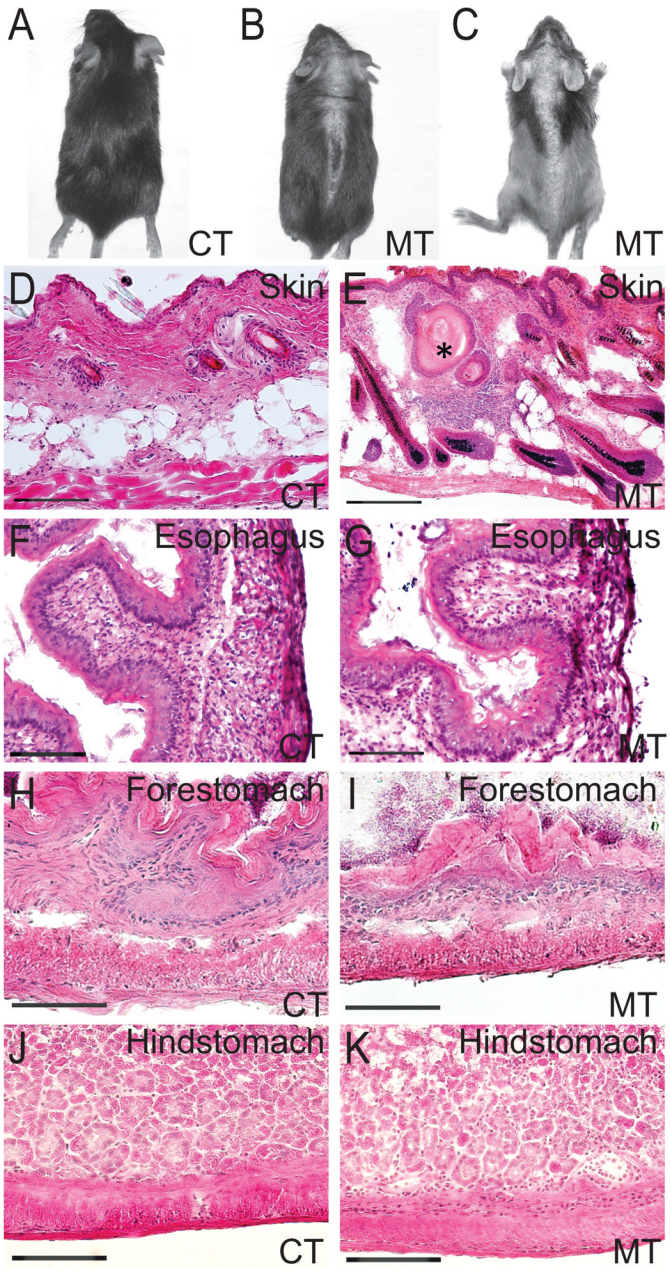

Figure 4. Megsin-Cre mediated deletion of Adam10 led to skin and stomach defects

Megsin-Cre/+, *Adam10*/+ mouse exhibits a progressive hair loss starting from the midline in both ventral and dorsal skins (A–C). A: Macroscopic appearance of control mouse, B–C: *Megsin-Cre*/+, $Adam10^{loxP/loxP}$ mouse at Week 3(B) and Week 8(C). Morphological changes in the conditional mutant skin were found (D–E). Adult *Megsin-Cre*/+, $Adam10^{loxP/loxP}$ mutant mice display keratin cysts (star in E). Sagittal sections of adult control and mutant mice showing hypoplasia of squamous epithelium in the forestomach of *Megsin-Cre/+,* $Adam10^{loxP/loxP}$ mouse (I), when compared to the control (H). No morphological changes were detected in the esophagus (F–G) or glandular hindstomach (J–K). Scale bar: 100 μm.