

## Generation of sRAGE<sup>high</sup> transgenic mice to study inflammaging

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### 1. ABSTRACT

The receptor for advanced glycation end products (RAGE) interacts with multiple ligands and transmits inflammatory signals from damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) to cellular programs. RAGE shares ligands with another group of PRRs, *i.e.*, Toll-like receptors. Such ligand-receptor promiscuity generates coordinated and complex signaling patterns that provide a basis for the development of multiple inflammaging diseases. Soluble RAGE (sRAGE) functions as a RAGE decoy that scavenges DAMP/PAMP ligands and dampens inflammatory signals. Epidemiological studies have shown that a lower level of circulating sRAGE is associated with metabolic syndromes including obesity, diabetes, hypertension, and subclinical brain disease. We hypothesize that an elevated level of circulating sRAGE serves to modulate systemic and low-grade chronic inflammation that often occurs in old age, and therefore minimizes the risk of inflammaging diseases. Consequently, a higher level of circulating sRAGE may improve the health-span of the organism. A newly generated transgenic mouse that has a higher level of circulating sRAGE and maintains normal expression levels of RAGE serves as a model to test this hypothesis.

### 2. INTRODUCTION

One of the prominent hallmarks of aging is the alteration of intercellular communication (1). Accompanying this aging-associated change is the development of low-grade and systemic pro-inflammatory phenotypes during aging, termed “inflammaging”, that many human chronic diseases are its sequelae. “Inflammaging” impacts multifarious cellular pathways and significantly influences the senescent and metabolic state of the cell and physiological health of the body in older age.

RAGE is a promiscuous, pattern recognizing receptor (PRR) that has a wide-range of ligands (2). Although majorities of the identified RAGE ligands, including HMGB1, S100 family members, glycosylated biomolecules, and DNA from necrotic host cells, are endogenous, recent studies have shown that ligands from pathogens, such as lipopolysaccharides (LPS) and DNA/RNA from pathogens that interact with canonical Toll-like receptors (TLRs), also interact with RAGE (3, 4). Such interactions trigger inflammatory signaling. Similarly, endogenous ligands that were initially found to interact with RAGE including HMGB1 and S100 family members have also been shown to interact with TLR family members (5). The cross-

reactions of ligands between TLRs and RAGE generate complex inflammatory patterns that are likely to be regulated in a spatial-temporal manner, and on a tissue-, and cell-type specific basis *in vivo*. Given the signaling complexity and ligand promiscuity among these PRRs, as well as their overlapping functions, targeted blocking of any specific PRR may not be sufficient to modulate systemic inflammation, and therefore may not significantly ameliorate the risk of developing aging-associated, chronic diseases.

RAGE signaling, and specifically the inflammation that follows, has been implicated in the development of multiple aging-associated diseases. In experimental settings, mice lacking RAGE are protected from developing diabetes, diabetic and non-diabetic cardiovascular complications, atherosclerosis, and various brain ramifications (6, 7). Under endotoxemia, RAGE-null mice also have lower inflammatory and pathological indexes and exhibit reduced organ and tissue damages and mortality (4). Like TLRs, RAGE signaling also has physiological functions and benefits including immune protection against pathogen infections and preservation of bone homeostasis (8, 9). RAGE is also required to protect the lung from developing fibrosis in older animals (10).

Because RAGE-null mice used in previous studies lack both RAGE and sRAGE, it is not entirely clear that the reported phenotypes are due to a lack of RAGE, or a lack of sRAGE, as the two major isoforms serve opposite functions. *In vivo*, sRAGE is generated via two mechanisms: alternative splicing of the *Ager* gene (11, 12), and proteolytic shedding of the cell surface RAGE (13-16). The shedding mechanism, until recently, was the major obstacle for the generation of sRAGE-null transgenic mice that could clarify the role of sRAGE *in vivo*. Previous studies have documented the benefits of sRAGE in various experimental settings (17, 18). Epidemiological studies seem to corroborate the beneficial roles of sRAGE observed in these animal studies (19-23). Such benefits are likely due to the pleiotropic ligand-scavenging functions of sRAGE that modulate systemic inflammation in response to various tissue injuries during an organism's life time. Additional evidence supporting sRAGE's benefits came from studies using recombinant sRAGE. It has been shown that injected recombinant sRAGE attenuates injury-elicited inflammation in the vessel and reduces arterial hyperplasia in carotid artery balloon injury models (18, 24). Administered sRAGE also inhibits formation and progression of atherosclerotic plaques in murine models (25). However, the systemic role of sRAGE in aging remains unclear. To test the hypothesis that a higher circulating level of sRAGE is beneficial during aging, we designed a new transgenic mouse model that has a higher level sRAGE in circulation while maintaining a perpetual RAGE expression level to

conserve the receptor's physiological functions. We hope that sRAGE<sup>high</sup> mice serve as a valuable model and tool for aging research.

### 3. MATERIALS AND METHODS

#### 3.1. Ethical statement

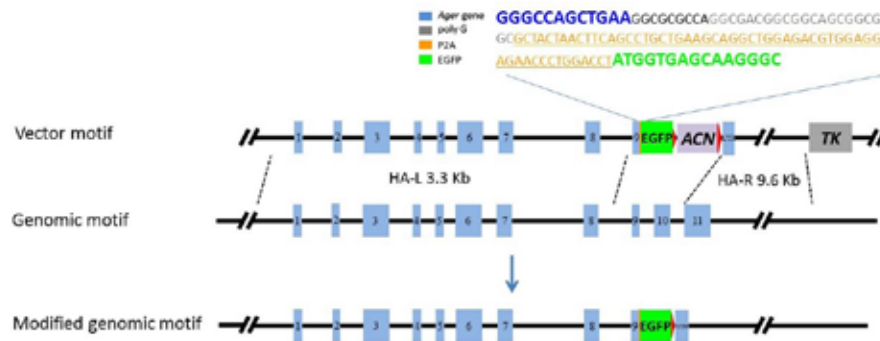
Mice were maintained at the National Institute on Aging (NIA) vivarium on ad libitum food (NIH-07 mouse/rat diet; National Institutes of Health, Bethesda, MD) with permanent access to filtered water. Breeding and all experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of NIA and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 3040-2, revised 1999).

#### 3.2. Generation of the sRAGE<sup>high</sup> targeting vector

The general cloning strategy is based on a protocol established by Wu *et al* (26), using a bacteriophage  $\lambda$  Red recombinase system. Specifically, a bacterial artificial chromosome containing a portion of murine chromosome 17 genomic sequence was obtained from Children's Hospital Oakland Research Institute and used to retrieve a 13.2. kb nucleotide fragment encompassing the *Ager* genomic sequence to the pStart-K vector. Exons 10 and 11 in the new plasmid were then replaced with a cassette containing P2A linked EGFP and self-excision ACN (tACE promoter-*Cre-Neo*, from pWS-TK3 vector) to generate a "gateway" compatible intermedial vector. Finally, the modified *Ager* was introduced into a vector containing HSV-TK (herpes simplex virus-thymidine kinase) marker to allow positive (neo) and negative (tk) selection when the targeting vector is introduced to the ES cells. Vectors pStart-K and pWS-TK3 used in the study were a gift from Dr. Mario Capecchi via Addgene. The final arrangement of the targeting vector is illustrated in Figure 1, and the joined sequences were sequenced to confirm their correctness.

#### 3.3. Generation of the sRAGE<sup>high</sup> transgenic mouse

The electroporation of the targeting vector to C57BL/6Ncr murine ES cells, screening of the positive clones, and microinjection of the ES cells to blastocysts of surrogate C57BL/6-cBrd/cBrd/Cr (albino) mice were performed in the Transgenic Mouse Model Laboratory, Frederick National Laboratory for Cancer Research, following the standard procedure. The resultant chimera mice were then crossed with the wildtype C57BL/6J and the black coat male progenies were used for congenic-assisted back crossing. sRAGE<sup>high</sup> HT mice with congenic biomarker-confirmed C57BL/6J genetic background were then used for breeding and



**Figure 1.** Generation and configuration of sRAGE<sup>high</sup> allele. After recombination, *Ager* promoter is activated in the sperm of the mouse, and the expressed *Cre* will excise the neo cassette to complete the transgenic configuration. The cleavage of the P2A peptide sequence generates the transgene sRAGE with 33 additional residues at the C-terminus, and 1 residue at N-terminus of EGFP. The blowup in the Figure shows the joined region that contains the C-terminus coding sequence of RAGE, poly-glycine and P2A peptide sequences, and the N-terminus sequence of EGFP. HA-L: homology arm-left; HA-R: homology arm-right, UTR: untranslated region.

expansion of the colony. The genotype of the wildtype (WT), sRAGE<sup>high</sup> homozygous (HO) and heterozygous (HT) mice were confirmed with genotyping using *Ager*- and transgene-specific probes.

### 3.4. Western blotting

Western blotting of the lung was performed as described previously (15, 27). Rabbit anti-RAGE antibodies (H-300) and anti- $\beta$ -actin antibodies were purchased from Santa Cruz Biotechnology.

### 3.5. Serum preparation and ELISA measurements

Blood samples were collected from eight weeks old mice, using retro-orbital bleeding and were left clotting at room temperature for 2 h. The samples were then centrifuged for 20 minutes at 2000 x g to obtain sera. An ELISA kit (R&D Systems Inc. catalog # MRG00) was used to measure sRAGE in serum in accordance to the manufacturer's instruction, using serum from RAGE-null mice as the negative control.

### 3.6. Statistical analysis

Data was expressed as mean  $\pm$  standard error of the mean (SEM).

## 4. RESULTS

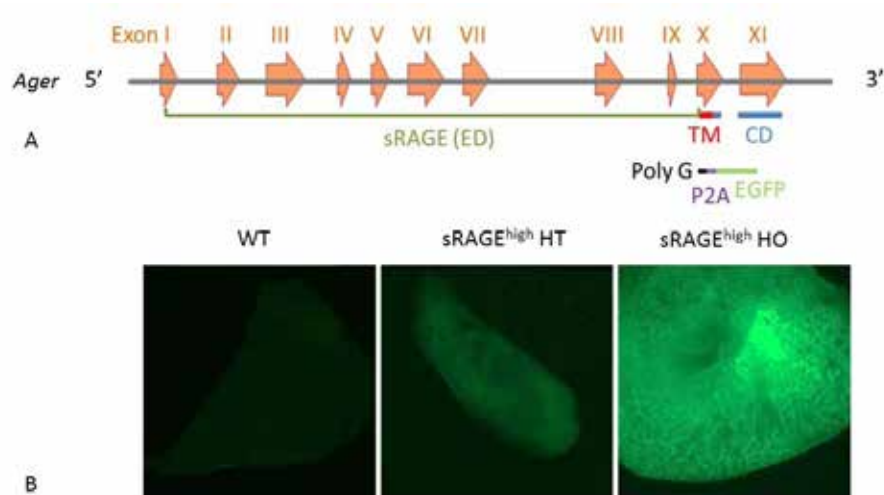
### 4.1. Rational design of an sRAGE<sup>high</sup> transgenic aging model

Our general strategy to generate the sRAGE<sup>high</sup> murine aging model was to delete the part of *Ager* gene encompassing the coding sequence of RAGE C-terminal portion, and then cross the transgenic mouse with the wildtype (WT) mouse to obtain hemizygous mice that express both transgene sRAGE

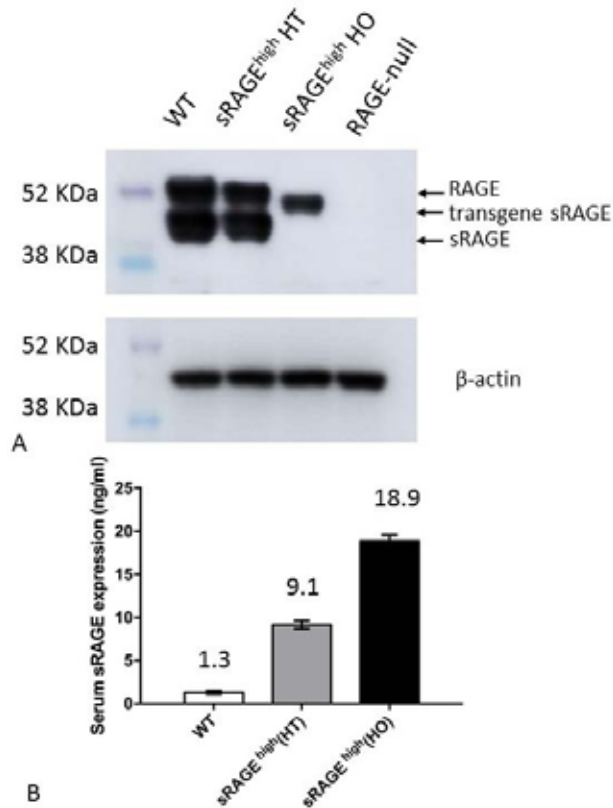
and RAGE. To assure that the transgene sRAGE is expressed in its natural cell- and tissue-types and can be secreted into the circulation, we replaced exons 10–11 of the *Ager* gene that encode the transmembrane and cytosolic signaling domains of the murine RAGE with a bicistronic control element 2A-linked EGFP expression cassette (Figure 1, Figure 2A). This design renders the transgenic animal RAGE-null, and the resultant sRAGE transgene being expressed in native RAGE-expressing cell types and regulated by the original *cis*-elements. The bicistronic arrangement also renders the RAGE-expressing cells fluorescent green in the transgenic mouse (Figure 2B), enabling *in vivo* and *in vitro* imaging applications. After backcrossing to C57B/6J genetic background, we crossed sRAGE<sup>high</sup> homozygous (HO) mice with C57B/6J mice to produce sRAGE<sup>high</sup> heterozygous mice (sRAGE<sup>high</sup> <sup>+/-</sup>, HT) as the designated model for aging studies. This model is also RAGE HT (RAGE<sup>+/-</sup>).

### 4.2. Characterization of sRAGE<sup>high</sup> hemizygous mouse

To test whether this hemizygous model satisfies our initial design, we examined the actual expression of RAGE/sRAGE in the lungs of sRAGE<sup>high</sup> HT mice, and the sRAGE level in their sera. Despite having only one genomic copy of full-length *Ager* gene, sRAGE<sup>high</sup> HT mice express a similar level of RAGE and naturally generated sRAGE in their lungs as that of the WT counterparts (Figure 3A). The level of transgene sRAGE in the lungs of sRAGE<sup>high</sup> HT mice is rather low, compared to sRAGE generated by shedding (*i.e.* natural sRAGE). In sera of sRAGE<sup>high</sup> HT mice, however, sRAGE levels are significantly elevated in comparison to that of the WT mice (Figure 3B), suggesting that transgene sRAGE is indeed released into the circulation. A higher level of circulating sRAGE appears not to affect the overall physiology of the transgenic mice, as both sRAGE<sup>high</sup> HT and HO mice develop normally into adulthood



**Figure 2.** Construction of sRAGE<sup>high</sup> mice. A. Schematic representation of murine Ager allele and the P2A bicronic expression cassette with EGFP that replaces exons 10 and 11. ED: extracellular domain; TM: transmembrane domain; CD: cytosolic domain. B. Representative fluorescence microscopic images of the whole lungs from WT, sRAGE<sup>high</sup> HO, and sRAGE<sup>high</sup> HT mice.



**Figure 3.** RAGE, sRAGE and transgene sRAGE expression in the lung and in serum. A. Western blotting of lysates from lungs of WT, sRAGE<sup>high</sup> HT, sRAGE<sup>high</sup> HO, and RAGE-null mice. B. ELISA analysis of circulating sRAGE in WT, sRAGE<sup>high</sup> HT, and sRAGE<sup>high</sup> HO mice (n= 3).

without apparent defective phenotypes. Under a regular diet, the bodyweight of adult sRAGE<sup>high</sup> HT mice, female and male, showed no statistically significant difference compared to WT counterparts (Table 1), suggesting a similar metabolic profile between the two groups.

## 5. DISCUSSION

Multiple elements influence the senescence state of the cell and impact the overall health- and life-span of an organism. Converging evidence suggests that

alteration of the intercellular communication including dysregulation of the immune system during biological aging leads to the development of immunometabolic disorders. Because of the ligand promiscuity between RAGE and TLRs, sRAGE can function not only as a RAGE decoy but also a modulator for several TLRs. This feature allows sRAGE to dampen inflammation elicited by a broad range of DAMP/PAMP ligands.

Recent studies have shown that TLR2 and TLR4 of the TLR family also produce soluble forms and sTLRs are found in body fluids such as plasma, saliva, and milk (28, 29). Like sRAGE, these sTLRs sequester their ligands, dampen inflammatory signaling of their respective receptors, and therefore modulate inflammation. Compared to sTLRs, circulating sRAGE as a systemic immune modulator has a few advantages. First, RAGE has a broader spectrum of ligands than any individual TLR. RAGE also shares ligands with several TLRs, thus allowing its decoy sRAGE to function as a broad TLR ligand sequester, modulating systemic inflammation elicited by not only RAGE, but also TLR ligands. Such ligand promiscuity and greater scavenging capacities of sRAGE affords broader protection against a wider range of DAMPs generated by various wears and tears during lifetime. Second, TLRs play prominent roles in the first line of biodefense against pathogen infections, and their innate signaling serves as the prerequisite for the development of adaptive immunity. Unlike TLRs, the biological functions of RAGE signaling are largely confined to sterile inflammation generated by tissue injuries and cell necrosis, pertaining to tissue repair and remodeling. Although it has been reported that RAGE-null mice are more susceptible to certain type of bacterial and viral infections (8, 30, 31), the protective role of RAGE in these events is likely to be indirect and complementary. Elevation of circulating sRAGE, hence, may not compromise the biodefense machinery, yet attain the benefits of circumventing chronic, low grade inflammation. Our design using sRAGE<sup>high</sup> HT, rather than HO, mice, should further eliminate the concern in this aspect, as the HT mice do not exhibit significant *Ager* haploinsufficiency and express adequate amount of RAGE (Figure 3A). This setting should also sustain other physiological functions of RAGE. Third, one group of primary ligands for RAGE are AGEs, which can be produced either endogenously or obtained from dietary sources (32). Dietary AGEs have been known to contribute to the development of multiple metabolic syndromes, and accumulation of AGEs in the body and in circulation exacerbates inflammation, in part, via RAGE signaling (33-35). Hyperglycemia and unhealthy lifestyles, including diets containing high AGEs and smoking, enhance AGEs generation. We speculate that a higher level of circulating sRAGE may reduce the health risk of AGEs that are either ingested or accumulate over a life-time. This idea can be tested by feeding the

sRAGE<sup>high</sup> HT mice with AGEs-enriched diets. Finally, sRAGE<sup>high</sup> mice can be crossed with other inflammaging disease models to study whether a higher level of circulating sRAGE can curtail or slow the progression of these diseases. For example, sRAGE<sup>high</sup> mice in *ApoE*-null genetic background can be employed to test whether a higher level of circulating sRAGE can prevent, reduce, or modulate high fat/high sugar diet-induced diabetes and other associated aging indexes. Because amyloid  $\beta$  is also identified as a RAGE ligand (36), sRAGE<sup>high</sup> mice can also be crossed with murine Alzheimer's models to study the impacts of sRAGE in the development of this detrimental aging-associated disease.

In WT mice, the level of sRAGE in the lung is quite high; yet sRAGE detected in plasma/serum is rather low (27) (Figure 3), suggesting that most of sRAGE generated by proteolytic shedding in the lung does not enter the circulation. Several studies also showed that sRAGE in the lung may serve distinct physiological functions from that in the serum including, paradoxically, to promote inflammation (37, 38). Epidemiological studies associate a low level of sRAGE in the serum with various ailments that often develop in older age (19-21, 23, 39), suggesting that unlike their counterpart in alveolar and bronchial cavities, sRAGE in circulation is likely to be beneficial. In addition, recombinant sRAGE administered via intra-peritoneal or intravenous injections shows anti-inflammatory benefits, whereas sRAGE administered by intratracheal injection exacerbates pulmonary inflammation (18, 24, 25, 37, 38). Using sRAGE<sup>high</sup> HT mice as an aging model may avoid the pitfall of reduced level of RAGE and sRAGE in the lung while exploit the benefits of higher level of sRAGE in circulation. The expressed transgene sRAGE in circulation apparently is native and functional, as it can be measured by ELISA, which relies on anti-RAGE antibodies that recognize native RAGE/sRAGE.

Despite its discovery a few decades ago, the regulation of the *Ager* gene is not well studied. Except in lungs, RAGE is expressed at low levels in other tissues and cell types, making quantitative studies of how *Ager* responds to stimuli difficult. We engineered the transgene bicistronic cassette using a 2A peptide coding sequence derived from porcine teschovirus-1(P2A), which confers high cleavage efficiency and can be used multicistronically (40). The near 1:1 co-expression of the transgene sRAGE and EGFP under the native *Ager* promoter makes it possible to monitor *Ager* gene regulation via measurement of the output of green fluorescence in live animals, and in isolated cells such as macrophages. These features of sRAGE<sup>high</sup> mice provide additional experimental options not only for aging studies, but also for mechanistic studies of RAGE signaling, leading to better understandings of the biology of this receptor.

Although an extension of health-span is not necessarily translated into a longer life-span, and vice versa, studies have been reported that associate higher levels of circulating sRAGE with human longevity (41). However, this observation has yet to be validated. Our data also showed that sRAGE<sup>high</sup> HT model has a similar bodyweight as that of WT counterpart (Table 1), suggesting that under a regular diet, a higher level of circulating sRAGE does not alter the metabolic profile. Because nutrients such as postprandial lipoproteins contribute to the inflammatory process (42, 43), it would be interesting to test whether under a high fat/ sugar diet or in an older age, whether sRAGE<sup>high</sup> HT mice have a healthier metabolic profile. We theorize that the pleiotropic scavenging capacity of sRAGE can be translated to holistic anti-inflammatory benefits during aging. Whether an elevated level of circulating sRAGE indeed minimizes the risk of inflammaging and elongates health- and life-span can be tested in our newly designed and generated sRAGE<sup>high</sup> HT model. This new tool is now available for researchers in the fields of aging, and aging-related diseases.

## 6. ACKNOWLEDGEMENTS

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