Supplementary Information for

Increased hyaluronan by naked mole-rat HAS2 improves healthspan in mice

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Supplementary Discussion

In the tissues of the naked mole-rat, HMW-HA is abundant and contributes to cancer resistance and possibly longevity of this exceptionally long-lived rodent[1,2]. Here we demonstrated that this evolutionary adaptation, unique to the naked mole-rat, can be "exported" to other species. The HMW-HA produced by naked mole-rat HAS2 gene conferred cancer resistance and increased lifespan of mice. nmrHAS2 mice accumulated HMW-HA in multiple major organs; these mice were resistant to spontaneous and chemically induced cancer and showed an extended median and maximum lifespan. nmrHAS2 mice also displayed improved healthspan including lower frailty scores and physical performance. This was accompanied by lower methylation age. The transcriptomes of aged nmrHAS mice had more youthful features when compared to the aging signature derived from the Tabula Muris Senis data[3]. The change conferred by nmrHAS2 was distinct from the transcriptomic changes triggered by known life-extending interventions including rapamycin, acarbose, growth hormone deletion, calorie restrictions and others. Interestingly, the nmrHAS2 altered the mouse transcriptome in a direction consistent with the transcriptomic signature of the long-lived species[4]. We therefore hypothesize that the presence of HMW-HA changes the transcriptome towards that of a longer-lived species (Fig. 4a).

What is the molecular mechanism by which HMW-HA extends mouse lifespan and healthspan? Importantly, due to the high conservation of the HAS2 gene, we do not believe that the naked mole-rat sequence *per se* was critical for the pro-longevity effect of nmrHAS2. Expressing mouse HAS2 gene showed the same protective effects *in vitro*. Rather, the increased production of HMW-HA was important. The most striking difference we observed between the transcriptomes of the aged nmrHAS2 mice and their littermate controls was the downregulation of multiple pathways related to inflammation in most tissues we analyzed. Beneficial effects of HMW-HA have recently been reported for muscle stem cells and adipose tissues where HMW-HA reduced inflammatory signaling and improved glucose homeostasis, respectively[5,6]. Interestingly, some of the effects were systemic in nature suggesting that even the tissues not displaying elevated HA levels can benefit from elevated HA elsewhere in the body[5,6]. Additionally, the plasma of aged nmrHAS2 mice also displayed reduced levels of multiple pro-inflammatory cytokines which indicates the systemic anti-inflammatory effect of HMW-HA. Age-associated chronic inflammation, so-called inflammaging, contributes to the pathogenesis of age-related

diseases[7,8]. It has been reported that individuals which have higher than age-average levels of inflammatory markers are more fragile and likely to be hospitalized[9].

The lifespan extension we observed in nmrHAS2 mice was significant but modest, while the healthspan improvement was more robust. While we observed strong expression of nmrHAS2 across all tissues, HA accumulation was modest and did not reach the levels observed in the naked mole-rat. This is likely explained by the high activity of hyaluronidases in mouse tissues[1,10]. In the naked mole-rat high level of HA is achieved by both robust synthesis and very slow degradation[1], yet in our model only the synthesis arm was modified. We hypothesize that if we were able to simultaneously attenuate HA degradation in nmrHAS2 mice we would achieve a greater lifespan extension.

We then discovered two independent pathways by which HMW-HA conferred an anti-inflammatory effect to the mice. First, HMW-HA had a direct immunomodulating effect on the immune cells of nmrHAS2 mice. Monocyte–macrophage lineage cells act as major effector cells in chronic inflammatory processes in aging-related diseases[11]. Macrophages are often divided into two subgroups: M1 and M2[12]. Aging may modulate M1/M2 activation and polarization. Sustained activation of M1 macrophage is linked to tissue dysfunction, while M2 macrophage promotes tissue homeostasis[13]. HMW-HA has been shown to prime macrophages towards the M2 state[14-16]. We also observed the same phenotype when we primed the bone marrow-derived macrophages from nmrHAS2 mice with *E. coli* lipopolysaccharide. BMDMs from nmrHAS2 mice showed a much higher expression of HAS2 gene, which may potentially help them to produce more HMW-HA and facilitates the alternative activation. HMW-HA was also shown to play an immunoregulatory role *in vivo* by binding to several immune cell types[17]. For instance, HMW-HA promotes the expression of FoxP3 in Treg cells by crosslinking CD44 which helps to maintain immunologic tolerance[18]. HMW-HA binds to CD44 and hSiglec-9 receptor which suppresses neutrophil extracellular trap formation and oxidative burst[19]. We observed that nmrHAS2 mice produced a lower level of inflammation after the LPS challenge which is consistent with previous reports that intraperitoneal injection of HMW-HA protects mice from LPS-induced sepsis[20].

The second, unexpected pathway, by which HMW-HA conferred its anti-inflammatory effect was through improved intestinal health. Aged nmrHAS2 mice were protected from leaky gut, and had healthier microbiomes. One of the major contributors to inflammaging is believed to be age-related deterioration of the intestinal barrier and translocation of gut bacterial products to

the bloodstream triggering an immune response[21]. Intestinal barrier failure is associated with aging-related systemic disorders such as obesity, metabolic disorder, brain dysfunction, and cancer[22,23]. Reduced thickness of the mucus layers was considered to be the cause of leaky gut[24]. It has been shown that the colonic mucus layer decreased in aging mice, suggesting an association with bacterial penetration and immune activation[25,26]. Interestingly, we found that elevated production of hyaluronan increased the number of mucin-producing goblet cells in both small intestine and colon, suggesting old nmrHAS2 are protected from leaky gut due to increased mucin formation. The increase in goblet cells in nmrHAS2 mice was significant, but very mild, and did not resemble a pathological over-proliferation of goblet cells observed in a disease state such as cystic fibrosis[27].

Studies in *Drosophila* showed that with aging intestinal stem cell (ISC) lose their stemness, are no longer able to differentiate into functional intestinal cells and undergo hyperproliferation associated with a loss of barrier function[28-30]. While the hyperproliferation of ISCs has not been unequivocally demonstrated in mammals[28,31], in mammals aging may be similarly associated with the loss of stemness by the ISCs. Indeed, it was found that the regenerative capacity of ISCs from old mice[31] and old human[28] was diminished *in vitro*. Consistent with these prior reports, we observed that ISCs from aged wild type mice formed fewer intestinal organoids. Remarkably, there was no such decrease observed for nmrHAS2 mice. Interestingly, the decline in the formation of organoids in the wild type mice could be rescued by adding HMW-HA to the culture media, indicating that the presence of HMW-HA promotes stemness of aged ISCs. It was reported that intraperitoneal injection of HA to mice promotes Lgr5+ stem cell proliferation and crypt fission through CD44 and TLR4[32,33]. Collectively, our results suggest that HMW-HA produced by nmrHAS2 transgene improves the maintenance of ISCs resulting in a healthier gut barrier during aging. Consequently, the healthier gut barrier function inhibits the shift of the gut microbiome towards proinflammatory commensals and reduction of beneficial microbes, slowing down the onset of inflammaging.

The resistance of nmrHAS2 mice to both spontaneous and induced cancer may be driven by both cell-autonomous mechanisms resulting from anti-proliferative signaling of HMW-HA through the CD44 receptor[34], and by its systemic anti-inflammatory effect[35]. Indeed, it was reported that HMW-HA blocks melanoma cell proliferation by signaling through CD44 to promote G1/G0 arrest[36]. HMW-HA also suppresses the growth of murine astrocytoma cell lines, glioma

and colon carcinoma xenografts[37]. HMW-HA has also been reported to reduce the migratory and invasive capacity of aggressive cancer cells[38,39]. On the other hand, chronic inflammation plays an important role in the development of cancer[7]. Many studies have shown that inflammatory cells can promote the occurrence and development of tumors by facilitating cancer cell proliferation, angiogenesis, and tumor invasion[40-42]. The anti-inflammatory properties of HMW-HA discussed above may potentially reduce the chronic inflammation during aging and prevent cancer initiation.

Additional pro-longevity effects of HMW-HA can be linked to its antioxidant and cytoprotective properties. Indeed, HMW-HA was reported to enhance cellular oxidative stress resistance[43,44]. Consistent with these observation fibroblasts from nmrHAS2 mice showed resistance to oxidative stress.

Supplementary references

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Supplementary Table legends
Supplementary Table 1. Primer sequences used for quantitative RT-PCR

Supplementary Table 2. CpG islands contribute to younger epigenetics of nmrHAS2 mice

Supplementary Table 3 correlation genes signatures Spearman Padjusted values

Supplementary Table 4. GSEA of enriched functions affected by nmrHAS2 and other interventions

Supplementary Table 5. Fisher functional enrichment of enriched functions affected by nmrHas2 and other interventions

Supplementary Table 1. Primer sequences used for quantitative RT-PCR

Gene	Forward primer	Reverse primer
actb	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGTAACAATGCCATGT-3'
HAS2	5'-GCCTCATCTGTGGAGATGGT-3'	5'-GAGTTTCTGTACATTCCCAGAGG-3'
Il1b	5'-CCGTGGACCTTCCAGGATGA-3'	5'-GGGAACGTCACACACCAGCA-3'
Il6	5'-AGTTGCCTTCTTGGGACTGA-3'	5'-TCCACGATTTCCCAGAGAAC-3'
tnfa	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	5'-TGGGAGTAGACAAGGTACAACCC-3'
arg1	5'-GCTCAGGTGAATCGGCCTTTT-3'	5'-TGGCTTGCGAGACGTAGAC-3'
Il12b	5'-AGACCCTGCCCATTGAACTG-3'	5'-GAAGCTGGTGCTGTAGTTCTCATATT-3'
nos2	5'-TTCACCCAGTTGTGCATCGACCTA-3'	5'-TCCATGGTCACCTCCAACACAAGA-3'
Il10	5'-GAGAGCTGCAGGGCCCTTTGC-3'	5'-CTCCCTGGTTTCTCTTCCCAAGACC-3'
Hyal1	5’-CATGCCTGAACCTGACTTCT-3’	5’-GTAGCAGTCAGGGAAGCCATA-3’
Hyal2	5’-CACCTGCCCATGCTGAAGGA-3’	5’-TCAGGAAAGAGGTAGAAGCC-3’