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Commentary on Some Recent Theses Relevant to Combating Aging: December 2014

Benjamin Zealley and Aubrey D.N.J. de Grey

IN THIS COLUMN, we continue the series, begun in issue 10(1), of surveys highlighting a small selection of recently completed doctoral theses with particular relevance to the fields covered by *Rejuvenation Research*.^{1–7} While it has become common for thesis work to appear in the general academic literature, it remains valuable to scan the thesis databases for important advances that one might otherwise overlook.

Antisense Reduction of the Protein Tau Attenuates Neuronal Hyper-Excitability and Permits Clearance of Intraneuronal Tau Accumulations *In Vivo*

Sarah DeVos, PhD, Washington University in St. Louis

The protein tau is a major contributor in some of the most prevalent neurodegenerative diseases, including the most common form of dementia, Alzheimer's disease (AD). As a member of the microtubule-associated protein family, tau is enriched in the axons of mature and growing neurons, although under certain conditions, it can become hyperphosphorylated and accumulate into toxic oligomeric species and aggregates. In the studies outlined here, we sought to directly target the protein tau using antisense oligonucleotides (ASOs) to reduce total expression of tau *in vivo* and assess if such a reduction could be therapeutically beneficial. To first test the feasibility of reducing tau in the adult animal, we identified ASOs that reduce endogenous mouse tau in the brain and found no effect on baseline motor or cognitive behavior. We then tested the efficacy of reducing murine tau in the context of hyper-excitability because aberrant neuronal excitability has been linked to AD pathogenesis, both in humans and in amyloid-beta-depositing mouse models. We found that mice with reduced tau had significantly less severe seizures than control mice, demonstrating that endogenous tau is indeed integral to regulating neuronal hyper-excitability. While the inducible models are sufficient to assess the roles of endogenous tau, non-transgenic mice do not develop tau aggregates. One of the main pathological AD hallmarks is the presence of tau inclusions, so to better test the effect of tau reduction on pathological tau species, we reduced human tau in a transgenic tauopathy mouse model that develops extensive tau pathology. Following treatment with a human tau ASO, not only did reducing human tau prevent additional tau aggregates from forming, it also allowed for a striking reversal of tau accumulations and hippocampal neuronal loss in aged tauopathy mice. Taken together, the safety of reducing endogenous tau in adult animals, the protective effect against neuronal hyper-excitability, and the ability to clear pre-existing tau aggregates, a tau-lowering therapy using ASOs may be a viable and strong therapeutic approach for those human patients with a detrimental hyper-excitability profile, tau inclusions, or even both.

Comment: *Pathological tau aggregates exist in the context of ongoing background synthesis and degradation of functional tau, but themselves accumulate only over extended periods of time, suggesting that even in aged tissue the mismatch between rates of accumulation and clearance is relatively small. Thus, it is not unreasonable to speculate that reducing production of new tau monomers would temporarily sway the biochemical equilibrium in favor of dephosphorylation and disaggregation (if this is not the case, then lysosomal modulation techniques will be required to hasten clearance⁸). This study investigates this possibility using antisense therapy: The introduction into a cell of a single-stranded oligonucleotide sequence complementary to one normally expressed therein, resulting in the subsequent suppression (or alternative splicing) of the native protein product. Although functionally related to the phenomenon of RNA interference, ASOs trigger a different endogenous mechanism—typically degrading their target via ubiquitous RNase H activity rather than through the relatively complex Dicer pathway, which operates only on 20- to 24-bp sequences. Combined with the smaller size of the therapeutic molecule for a given sequence—small interfering RNAs must be double-stranded, a significant consideration in delivery—this has the potential to streamline clinical translation. In practice, the ASO must still be extensively chemically modified to protect it from ubiquitous nuclease activity, to permit transport across cell membranes, and to target it to the correct cell population (a range of obstacles that have led some researchers to pursue entirely artificial silencing methods⁹). Despite these challenges, there are*

over 40 antisense and RNA interference-based therapies currently in clinical trials or on the market, a testament to the power of the approach. The anti-tau ASO introduced here appears both safe and highly effective in the models examined. Although it seems optimistic to expect profound neuronal recovery in advanced human tauopathy patients—where lost cells may have been absent for an order of magnitude longer than in transgenic mice, and are thus more likely to require outright replacement¹⁰—we are very eager to discover whether the agent is sufficiently well tolerated and efficacious in humans to permit its use as a prophylactic in mild or even pre-clinical cases.

Discovery of Novel Serum Biomarkers for Diagnosing and Staging Alzheimer's Disease

Dipti Shah, PhD, Brigham Young University

Alzheimer's disease (AD) is an untreatable neurologic disease affecting more than 5 million Americans, most over 60 years of age. Protein plaques and neurofibrillary tangles typify AD brain pathology and are thought to cause the progressive dementia and brain shrinkage observed in AD. Currently, there are no methods to diagnose the disease at a time before damage becomes irreversible.

Biochemical tests for AD using cerebrospinal fluid analysis or neuroimaging are not yet sufficiently sensitive and specific, and they are invasive. This points to a need for a more easily applied and more sensitive diagnostic test. Although the gross anatomical changes are localized to the brain, AD is likely to involve changes throughout the body. As a result of this, changes in the abundance of certain biomolecules present in the circulation system are likely to occur. Consequently, a serum proteomics approach able to measure such changes, when applied to AD, would likely find quantitative changes in relevant molecules that can help diagnose the disease correctly, ideally early in the disease process.

The goal of this work was to discover and validate novel diagnostic serum biomarkers for AD. For biomarker discovery and validation, we used a novel serum proteomics approach involving reversed-phase capillary-liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry. Our samples were protein depleted, which helped us survey low-molecular-weight species in the serum without ion suppression from larger proteins like albumin. We were able to observe more than 8000 molecular species in a single run. The overall project was comprised of four studies: (1) Discovery of novel potential serum AD markers, (2) blinded validation of diagnostically promising biomarkers found in the initial study, with their further chemical identification, (3) exploring gender-based serum AD biomarkers, and (4) discovery of biomarkers that distinguish early- versus moderate-stage AD.

In the first study, the approach found 38 significant ($p < 0.05$) biomarkers and 21 near-significant ($p = 0.05$ – 0.099) biomarkers. On using the forward selection approach, we built multi-marker panels with specificities and sensitivities higher than 80%.

The second study reports on a blinded validation study that was performed on a new set of serum samples. We focused on the 13 most promising AD biomarkers found as part of the initial study. We successfully validated four of the biomarkers that showed highly significant statistical p values. As part of this study, research was conducted to identify these four biomarkers, which was accomplished using tandem mass spectrometry with fragmentation experiments.

The third study used data from the initial study but looked at gender-specific biomarkers. We found 31 significant and near-significant serum AD biomarkers for women—16 for men and 25 that were gender independent. Multi-marker panels of AD biomarkers for women or men had sensitivities of $>60\%$ and specificities $>85\%$.

In the fourth study, cases with moderate AD were compared to cases with very mild or mild AD to find novel biomarkers that could be used for staging. We found 44 significant and near-significant biomarkers that were quantitatively different between mild and severe AD. In conclusion, we were successful in accomplishing the goal of this work of finding, validating, and identifying novel serum biomarkers that diagnose AD.

Comment: *Most age-related damage, even if effectively irreparable in situ, can at least in principle be remedied by replacement of the entire tissue or organ affected with newly engineered tissue. This approach relies fundamentally on the assumption that a given body part is functionally equivalent between individuals, and hence breaks down in the case of the brain, where not only the types of cells and their environment, but also their specific interconnectivity, is absolutely critical to function and vulnerable to disease processes.¹¹ Consequently, the goal in treatment of neurodegenerative conditions must be to not merely arrest disease progression, but to avert it altogether. Accomplishing this will be much simplified by a clear picture of the course of disease development before the point at which symptoms appear. Acquiring such a diagnostic capacity, especially by means amenable to scaling up to population screening, has proven extremely difficult, reflecting in part the fact that a large proportion of the adult populace has some degree of pre-Alzheimer pathology, despite the lack of recognition of this unfortunate biological truth by medical regulators,^{12,13} as well as the complexity of the disease process. Given the psychological impact of a misdiagnosis, the proteomic approach used here does not reach sensitivities and specificities high enough for immediate clinical use. However, it represents an important proof of concept, and leaves us optimistic that folding in data from lipidomic, glycomic, and other “big data” analyses may yet yield an outpatient blood test capable of quantifying pre-neurodegenerative decline. The extent to which such a metric would simplify the process of obtaining regulatory approval for prophylactic therapies should not be underestimated.*

Economic Analysis of Preventive Care Utilization among Older Adults

Boon Ng, PhD, Wayne State University

This dissertation seeks to examine the economic determinants of the use of preventive services among older adults. It contains two studies that focus on the effects of public health policy and health shocks on the initiation of use of preventive services among older adults.

In January, 2005, Medicare began covering a one-time initial preventive physical examination (IPPE), also called a “Welcome to Medicare” visit, for new beneficiaries. This benefit was only available during a beneficiary’s first 6 months after enrolling in Part B. The first study examines the effects of covering an IPPE on the use of mammograms, breast self-exams, Pap smears, prostate cancer screenings, cholesterol screenings, and flu vaccines among beneficiaries new to Medicare Part B. Using data from the 1996–2008 Health and Retirement Study (HRS) and the RAND HRS, I estimate multivariate logit models to quantify the effects of Medicare coverage of an IPPE on the utilization of each of these preventive care services. The findings indicate that, among both men and women, the introduction of Medicare IPPE coverage during a beneficiary’s first 6 months under Part B did not increase the utilization of any of the preventive services examined.

Although about 70% of older adults will have one chronic condition and 50% will have more than one chronic illness such as heart disease, cancer, stroke, etc., only 25% of adults ages 50–64 and fewer than 40% of adults ages 65 and older are up to date on recommended preventive health care services. The second study evaluates whether new information, acquired through the occurrence of unexpected adverse health events, leads an individual to begin using preventive care services. Using data from the longitudinal Health and Retirement Study (HRS) and the RAND HRS, multivariate logit models are estimated to model the dynamic effects of exogenous health shocks on the initiation of use of mammograms, breast self-exams, Pap smears, prostate cancer screening, cholesterol tests, and flu vaccinations. Findings reveal that among adults with a history of not using preventive care, an unexpected adverse health event often spurs them to begin using such services. Among women ages 40 and older, those who experience an adverse health shock are 1.87 times more likely to begin getting mammograms, 1.48 times more likely to begin getting Pap smears, 1.79 times more likely to begin getting cholesterol tests, and 1.46 times more likely to begin getting flu vaccinations. Among men ages 40 and older, those who experience an adverse health shock are 2.24 times more likely to begin getting prostate cancer screenings, 2.75 times more likely to begin getting cholesterol checks, and 1.64 times more likely to begin getting flu vaccinations. These findings provide strong evidence that people change their health behaviors in positive ways following the occurrence of a negative health experience.

Comment: *Aging is a continual process of damage accumulation, masked for decades by the body’s not inconsiderable reserve capacity. The diseases associated with advanced age manifest when this damage crosses some critical threshold, overwhelming the body’s repair systems and leading to visible and often irreversible pathology. There are multiple qualitatively distinct but functionally inter-related classes of aging-related damage, and consequently such conditions are usually multi-factorial and difficult to understand, let alone treat. Rather than attempting to arrest the decline of a system already teetering on the brink of failure, the field of rejuvenation biotechnology aims to repair the accumulated damage of aging, rendering the body biologically “younger” and more robust, while (in principle indefinitely) delaying the onset of disease. The success of this endeavor will depend in no small part on educating the general public on their right to good health,¹⁴ and the importance of receiving preventative medical care, to a much greater extent than has been the case in any society to date. This work presents troubling news on that front. The availability of free health checks is found to not stimulate engagement with preventative care services, implying that even were tests similarly available to quantify pre-clinical age-related damage, uptake thereof may require more elaborate encouragement than simply eliminating any financial barrier. This is likely to be due at least in part to the aversion experienced when considering seriously the possibility of future ill health or death, a necessary prerequisite to actively seeking out preventative care. The increase in service utilization following a health shock does at least suggest an opportunity for intervention, targeting recently diagnosed patients, who have recently been forced to confront their mortality, for broad-spectrum health checks. The era of personalized medicine will offer such patients new opportunities to engage more effectively with their own care, even in cases of currently incurable disease.¹⁵*

MagA As a Genetic MRI Reporter for Longitudinal *In Vivo* Stem Cell Monitoring

In Cho, PhD, Emory University

The ability to longitudinally monitor cell grafts and assess their condition is critical for the clinical translation of stem cell therapy in regenerative medicine. Here, we investigate feasibility of using MagA as a genetic magnetic resonance imaging (MRI) reporter for longitudinal stem cell graft *in vivo*. MagA is a bacterial gene involved in forming iron oxide nanocrystals. MagA expression was regulated by the Tet-On switch, hence reducing cytotoxicity

and allowing inducible monitoring by supplementing doxycycline (Dox). We established a mouse embryonic stem cell (mESC) line carrying Tet– MagA (mESC-MagA) by lentivirus transduction. Expression of MagA in mESCs resulted in significant changes in transverse relaxation rate (R_2 or $1/T_2$) *in vitro*. mESCs with and without MagA (mESC-MagA and mESC-WT) were grafted to the striatum of mice brains and longitudinally monitored *in vivo* using MRI with “ON” (Dox+) and “OFF” (Dox–) conditions. Intracranial mESC-MagA grafts generated sufficient T2 and susceptibility weighted contrast at 7T, allowing for visualization of the graft by MRI longitudinally in controlled “ON” and “OFF” fashion upon induced expression of MagA by administering Dox in diet. Our results suggest MagA can be used to monitor cell grafts non-invasively and longitudinally by repeated induction, enabling the assessment of cell graft conditions.

Comment: *Magnetic resonance imaging (MRI) is frequently the optimal choice for both clinical and research purposes, offering excellent resolution, tissue penetration, and soft tissue discrimination without the use of ionizing radiation. For many applications contrast enhancement is required to distinguish between similar cells. To date, this has typically been achieved through the use of injected or ingested chemical agents containing gadolinium, iron, or manganese, all of which shorten the magnetic relaxation time of nearby protons and thus generate a detectable signal variation. However, even intravascular administration introduces significant confounding variables. Uneven distribution of the contrast agent through the body, unpredictable rates of uptake amongst target cells, and clearance of the agent from the circulation are just a few factors that limit the data that can be acquired. MagA, a bacterial iron transporter gene isolated from Magnetospirilla species, was recently confirmed to be sufficient for the production of MRI-detectable iron nanoparticles within living mammalian cells,¹⁶ and thus constitutes the first MRI reporter suited to in situ contrast generation in cells of interest. This exciting work provides one example of a clinical application—tracking a stem cell graft, including the entire lineage of progeny cells—a procedure that seems likely to also be of great value in developmental biology, where it will facilitate the visualization of entire stem cell lineages in a fashion previously possible only in small/transparent organisms or by dissection. Another putative application would be in immunotherapy, where the tracking of newly introduced immune cells could allow clinicians to rapidly locate their targets throughout the body. The 7T field strength used in this work is considerably stronger than that generated by typical clinical models (around the 1.5T mark). It remains to be seen whether engineering of the MagA gene can further improve its function to the point where widespread clinical application would become feasible in advance of the general adoption of higher-field imaging hardware.*

Targeting Cancer Metabolism with Ketosis and Hyperbaric Oxygen

Angela Poff, PhD, University of South Florida

Cancer cells exhibit an abnormal metabolic phenotype characterized by glycolysis and lactate fermentation in the presence of oxygen, a phenomenon known as the Warburg effect. This dysregulated metabolism plays an important role in every aspect of cancer progression, from tumorigenesis to invasion and metastasis. The Warburg effect is a common phenotype shared by most, if not all, cancer types. It is especially prominent in metastatic tumors, which are notoriously resistant to treatment and responsible for the majority of cancer-related deaths. Thus, metabolic therapies that target the Warburg effect could offer novel therapeutic options for most cancer patients, including those with aggressive or late-stage cancers. The ketogenic diet is a high-fat, low-carbohydrate diet that induces a physiological state of nutritional ketosis—decreased blood glucose and elevated blood ketones. It has been investigated as a cancer therapy for its potential to exploit the Warburg effect by restricting glucose availability to glycolysis-dependent tumors, and has been reported to slow cancer progression in some animal models as well as in anecdotal reports and small clinical studies in humans. Interestingly, there is some evidence that the elevation in blood ketones induced by the ketogenic diet contributes to its anti-cancer effects, suggesting that ketone supplementation could possibly inhibit cancer progression on its own. Rapid growth outstrips a tumor’s ability to adequately perfuse its tissue, creating regions of tumor hypoxia that exacerbate the Warburg effect and promote a malignant phenotype. Hyperbaric oxygen therapy is the administration of 100% oxygen at elevated barometric pressure. It supersaturates the blood with oxygen, increasing its diffusion distance into the tissues, and can therefore be used to increase intratumoral pO_2 and reverse tumor hypoxia. Here we present evidence that the ketogenic diet, ketone supplementation, and hyperbaric oxygen therapy work individually and in combination to slow progression and extend survival in the VM-M3 model of metastatic cancer. This study strongly suggests that these cost-effective, non-toxic metabolic therapies should be further evaluated in animal and human studies to determine their potential clinical use.

Comment: *Economically and efficiently tackling cancer in all its myriad forms requires a focus on aspects of the disease that are at least relatively consistently observed. Such therapies may be less effective in isolation versus any particular strain of tumor than those targeting its specific genetic or metabolic abnormalities (although there is at least one promising exception already entering human trials¹⁷), but have the potential to be used as an accessory in a broad range of pathological scenarios, justifying their investigation and refinement. The metabolic conditioning techniques discussed in this thesis are excellent examples of such supplementary therapies. Further clinical options derived from current work on the somatic cells of the tumor niche—which unlike the tumor proper retains a stable (if often abnormal¹⁸) genetic environment, facilitating manipulation—are likely to be similarly widely applicable, but*

are unlikely to be as cheap or as readily implemented. We note that the recent swing in consensus nutritional opinion regarding dietary carbohydrates and fats suggests that encouraging more general use of ketogenic diets could have significant public health benefits in non-cancer patients as well.

The Role of Cortical and Cancellous Bone Quality on Vertebral Fragility

Brian Bradke, PhD, Rensselaer Polytechnic Institute

A serious health concern of aging populations is the increased risk of non-traumatic fracture caused by age-related changes in bone's structure and morphology. Thinning bone caused by asymmetric bone resorption, diagnosed as osteoporosis, is commonly the focal point for age-related fracture risk. Although the majority of orthopedic research is conducted on decreased bone quantity, changes in bone's quality are as equally impactful on the fracture mechanics of bone and are largely overlooked. One of the most prevalent modifications in aging bone is the accumulation of advanced glycation end products (AGEs) caused by non-enzymatic glycation (NEG). AGE content in bone increases with age and has a deleterious effect on bone's material properties.

Vertebral fractures account for more than half of all osteoporotic fractures and correlate to increased mortality and morbidity. To define an objective fracture risk indicator, interactions between cortical and cancellous constituents of vertebrae have been widely studied. Notwithstanding, some disparity remains over the exact fracture modality within the vertebrae. Despite known limitations, bone mineral density (BMD) remains the standard for assessing vertebral fragility. As alterations in bone's organic matrix have a marked impact on the tissue's material properties, it is imperative to elucidate the effects of non-enzymatic glycation on load-bearing ability of the vertebrae's constituent parts.

Therefore, the objective of this doctoral research is to combine biochemical, mechanical, and computational methods to: (1) Better characterize the load-sharing role of cortical and cancellous bone within the vertebrae, (2) determine the effect of AGE accumulation on load sharing within the vertebrae, and (3) validate the feasibility of reversing the effects of NEG-mediated AGE cross-linking using *n*-phenacylthiazolium bromide.

The results presented herein provide a better understanding of load sharing and fracture within the vertebrae. They also reinforce the degenerative nature of age-related changes within bone's organic matrix, stressing the importance of considering quality as well as quantity of bone when defining fracture risk. To reverse the effects of NEG, a novel compound is proposed and verified as a possible therapy for the accumulation of AGEs within bone's structure. Finally, a computational model encompassing a damaged-plasticity material definition is vetted as a predictive tool for assigning fracture risk. When combined, this work may provide a baseline for the development of much needed fracture risk assessment tools to diagnose and prescribe necessary treatments for the aging skeleton.

Comment: *The relationship between AGEs and tissue biomechanics is an area of highly active investigation. Long-lived cross-links such as glucosepane modify the collective properties of ensembles of proteins, leading to a gradual loss of high-level function—elasticity in the vasculature, transparency in the eye, and so forth. Fortunately, such cross-links are chemically atypical, raising hopes for their selective degradation.¹⁹ Cross-linking of collagen fibers in bone leads to increased brittleness and fracture risk and is thought also to interfere with resorption. Bone left unrecycled for too long is, of course, likely to be subject to other biochemical insults, including further glycation. This process is most damaging in the cancellous bone, where the bone mineral matrix is less dense and the tissue thus more dependent for its structural properties on protein biomechanics. Unfortunately for us, this includes the core of the spinal column. *n*-Phenacylthiazolium bromide (*n*-PTB) is a precursor to the drug candidate ALT-711, a proposed cross-link breaker²⁰ that has experienced mixed results in clinical trials. We are glad to see that further development continues on this family of molecules despite initial setbacks.*

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