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Review Article

Cancer telomeres and white crows

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Abstract: This mini-review article discusses past and present prostate-focused research on telomere and telomerase biology conducted at Johns Hopkins, through the eyes of a Donald S Coffey trainee. Included are past discoveries of abnormalities in telomere biology in the context of prostate cancer and its pre-malignant precursor prostatic intraepithelial neoplasia (PIN); the finding that telomerase activity is androgen-regulated in the prostate, and the potential role of telomerase in prostate epithelial stem cells. Also reviewed are more recent results showing that in situ telomere length measurements in patient tissue specimens may have utility in risk assessment and as a prognostic biomarker. Highlighted throughout the article are some of the training and mentorship approaches employed by the late Dr. Coffey, former Director of Urologic Research at the Brady Urological Research Institute, which inspired new research ideas, team science, and discovery.

Keywords: Telomeres, telomerase, chromosomal instability, cellular immortality, Hayflick limit, prostatic intraepithelial neoplasia, prostate cancer, androgen regulation

Introduction - a tribute to Don S. Coffey

It is an honor to be writing an article for inclusion in this special issue of the American Journal of Clinical and Experimental Urology, honoring the late Dr. Donald S. Coffey. The passing of a dear friend is always a sad affair, but when that friend is also a revered mentor and colleague, and one of the finest human beings one has ever met, the loss felt is compounded proportionally. These past months have been particularly tough on us here at Johns Hopkins, Dr. Coffey's home institution. The winter this year in Baltimore has seemed especially long and dark; the winds colder than usual. However, significant compensation has come in the form of the mutual support and love we, his friends and colleagues, provide one another; in the process, keeping Don's memory and spirit alive in each of us.

Human destiny

I first encountered Don through my former mentor, Dr. David Shortle, a faculty member in the Hopkins Department of Biological Chemistry. Dave had done a research rotation in Don's lab several years before, during his M.D. Ph.D. training, and had since established his own research lab, where he was focusing on the protein folding problem. At that time, I was working as a technician in Dave's lab as a prelude to graduate school. Dave often spoke admiringly about Don, and one of the few pictures adorning the walls of his relatively Spartan office was an enthusiastically signed cover of the Johns Hopkins Magazine featuring Don's picture. Dave introduced me to Don's annual Human Destiny lecture, a tour de force presentation that Don delivered to the entire School of Medicine every St. Patrick's Day, a date chosen to honor of his Irish heritage. I had never seen anything like this before. Over the course of a couple of hours, Don gave a high-energy talk brimming with infectious enthusiasm that integrated physics, chemistry, cosmology, biology, human evolution, religion, good and evil, and human creativity. It was an amazing feat to witness; exceptional, even among the backdrop of other brilliant talks one routinely encounters at top

tier universities. Clearly here was a man with insatiable curiosity, someone who thought deeply about important issues and asked the "Big Questions", excitedly sharing his thoughts and knowledge with others.

A couple of St. Patrick's lectures later, I entered the BCMB doctoral program at Hopkins, in which I had initially planned to work in basic structural biology. However, my plans changed when my father was diagnosed with esophageal cancer, to which he quickly succumbed. Not only was this emotionally devastating, but during the course of his illness, I encountered both my own deep ignorance regarding this disease we call cancer, as well as the field's inability to effectively treat advanced cancers, a problem that still exists decades later. This compelled me to switch my focus to cancer research, which brought me, ultimately, to Don's lab. To this day, I feel incredibly blessed to have been afforded the opportunity to work under Dr. Coffey; "The Chief", as he was widely known.

Cancer cell immortality

Don Coffey was a master at doggedly pursuing fundamental questions that arose from seemingly basic observations, such as, "Why are cancer cell nuclei so morphologically abnormal?" His guiding mantra in such situations was, "If this is true what does it imply?" - a question which ultimately led to his fundamental discovery of the nuclear matrix [1]. Another primary characteristic of cancer cells, distinguishing them from their normal counterparts is their seemingly unlimited capacity to divide, the so called, "immortal phenotype" [2]. In the early 1960s, pioneering work by Leonard Hayflick and Paul Moorhead showed that normal human cells have a finite division capacity, overturning the then reigning dogma that cells had innate immortality, when grown ex vivo under the optimal conditions [3, 4]. Through elegant experiments, Hayflick and Moorhead convincingly demonstrated that this was, in fact, not the case. Instead, through mechanisms then unknown, cells somehow kept track of the number of times they had divided; ceasing their division once a set number had been achieved (the so-called "Hayflick limit"). It has been proposed that this stringent restriction on clonal expansion represented an evolved block to the outgrowth of cancers [5]. These observations implied that the cell possesses a mitotic counting element that keeps track of cell divisions, and that cancer cells somehow thwart this process. Precisely how cancer cells were able to overcome this block remained yet to be discovered.

We now know that what the cell monitors as the indicator of cell division are the telomeres - repetitive DNA sequences at the termini of each of our chromosomes [6, 7]. DNA polymerase is unable to fully replicate the very ends of linear DNA molecules, termed the "end-replication problem" that was simultaneously recognized in the 1970s, both by James Watson in the United States, and Alexi Olovnikov in Russia, upon the elucidation of the enzymatic mechanism of DNA polymerase [8, 9]. In the 1980s, the vertebrate telomere DNA repeat sequence (TTAGGG) was identified, thus allowing for the monitoring of telomere kinetics during cell division via standard molecular biology techniques, such as Southern blotting, and later by quantitative PCR [10-13]. Using such techniques, it was found that, as predicted by the end-replication problem, telomeres in normal cells progressively shortened, at a rate of approximately 50-100 base pairs per cell division, during multiple rounds of cell replication [12, 14]. In 1998, Bodnar and colleagues elegantly demonstrated that telomere shortening served as the replicative counting mechanism used by normal cells to trigger the Hayflick limit, through experiments in which telomerase was expressed in telomerase-negative normal human cells growing in culture. When telomerase was introduced prior to the Hayflick limit, it halted telomere attrition and allowed the cells to grow indefinitely [15]. Cancer cells possessed very short telomeres, significantly below the length which would instigate the Hayflick limit in their normal cellular antecedents; and yet, despite their continued cell division, cancer cells were somehow able to maintain their telomere lengths, albeit at abnormally short levels. [16] It was at this point that I joined Don's lab, and we decided that I should study this phenomenon.

Telomerase activity in prostate cancer

Progress in the field at this time was rapid. I had only just begun formulating my own research project when, while waiting for a first year molecular biology class lecture to begin one day, I noticed a curious headline in the newspaper that someone in front of me was

reading, "Cancer immortality enzyme discovered". Chris Counter had discovered that the enzyme telomerase was active and maintaining telomere lengths in human B lymphocytes that had been transformed and immortalized by Epstein-Barr virus infection, and he further detected telomerase activity in ascites fluid samples from human ovarian cancer patients [17, 18]. Telomerase had been discovered several years earlier by Carol Greider, working with the ciliated protist Tetrahymena thermophila, in Elizabeth Blackburn's lab at UCSF; work which subsequently earned them the Nobel Prize [19, 20]. This work showed that telomerase functioned as a processive reverse transcriptase, utilizing an integral RNA subunit that contained the telomere repeat sequenced sequence as a template for telomere DNA repeat addition through reverse transcription. This knowledge, in turn, led to the development of a PCR-based assay for the detection of telomerase enzymatic activity in cell or tissue extracts [21]. In Don's lab, we quickly looked to see if the same phenomena identified in the ovarian cancer patients were at play in prostate cancer, as prostate cancer and other prostatic diseases were the primary focus of Don's lab at the Brady. Working closely with a talented clinician, Hans Sommerfeld, visiting from Germany, we were able to show that prostate cancer cells had abnormally short telomeres and that the vast majority of extracts from prostate cancer patient tissues possessed telomerase; in stark contrast to normal prostate cells and tissues [22]. We were also able to profile the benign proliferative disease, benign prostatic hyperplasia (BPH). We found that, in matched samples from the same patients; unlike prostate cancer, BPH did not display abnormal telomere shortening despite the fact that BPH is also a disease characterized by deregulated proliferation [22]. Also in contrast to prostate cancer, BPH lacked detectable telomerase activity; thus, cells in BPH were apparently not immortalized. To explain these differences between cancer and BPH, we hypothesized that prostate cancer, being a clonal disease, had undergone many more cumulative rounds of cell division in order to produce a clinically detectable tumor; whereas, despite production of comparable tumor masses, BPH was not of clonal origin, representing instead a hyper-proliferation of many benign cells in concert, thereby avoiding noticeable telomere loss and also avoiding the concomitant selective pressure for activating telomerase [23]. However, the question yet remains unresolved, as a recent report suggested that the BPH telomere phenotype results from putative telomerase-positive prostate epithelial progenitor cells in the basal epithelial cell compartment [24]. In order to explain our seemingly paradoxical findings in prostate cancer; that prostate cancer cells possessed short telomeres plus active telomerase, we hypothesized that, early during tumorigenesis, telomerase was either not present, or was present, but at insufficient levels to compensate for the ongoing telomere attrition occurring during clonal expansion of the malignant clone. We went on to show that in prostate cancer, telomerase expression was under the control of androgen-signaling, and that androgen withdrawal from prostate cancer cells growing in vitro resulted in rapid and profound suppression of telomerase expression, which could be rapidly reversed upon androgen restoration. Loss of telomerase via continued androgen withdrawal, in the androgen-sensitive prostate cancer cell line LNCaP, led to progressive telomere shortening, dramatically increased levels of chromosomal instability and interestingly, accompanying increases in gross abnormalities of nuclear structure, the common hallmark of cancer which Dr. Coffey had long been fascinated by [25].

Legal pads, leading questions, and the prostate stem cell

Another area of longstanding interest in Don's lab, with clear prostate cancer relevance, was that of the normal androgen-regulation of cell division, tissue development, and cell differentiation in the prostate gland. Don had a wonderful personalized Socratic method he would often use to guide and teach his trainees. I clearly remember one such episode where he led me to an understanding of the value of manipulating androgen levels in vivo for studying prostate biology. He began, in typical Don Coffey fashion, by asking a provocative leading question, "Is there any place in the body where you can turn DNA synthesis on and off at will?" The only thing I could think of at the time was that, perhaps, if some sort of infection were induced, then some cells of the immune system might proliferate, which, tangentially, led to an entire side discussion on the functioning of the immune system, the unique role of DNA rearrangements in generating diversity (something that also plays a role in cancer cell diver-

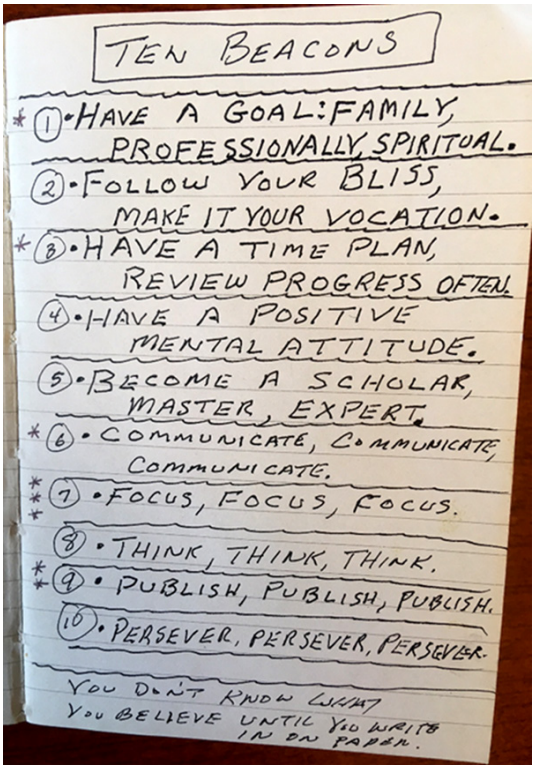

Figure 1. A kindly note from Don Coffey with encouraging personalized advice to the author during his graduate student training. Trainees received such cherished notes from "The Chief" on a regular basis.

sity), and the theoretical potential of immunotherapy as an adaptive tool to fight an evolving disease such as cancer (at last beginning to bear significant fruit with the recent development of checkpoint blockade therapies). Eventually, we settled back to the original question, and Don outlined to me (again, largely through the use of guiding questions) that, following castration of a male animal, the prostate rapidly involutes (leading to another digression on the process of apoptosis in development and disease), and that upon reintroduction of testosterone, DNA synthesis is rapidly initiated, with the prostate quickly re-growing to its initial mass (another digression into the question of how normal organ and tissue homeostasis is maintained!). Through this long, wide-ranging and fascinating discussion, Don pointed out that this represented a unique in vivo experimental system, and that one could, in fact, repeatedly perform such cycles of androgen withdrawal/gland involution - androgen restoration/gland regrowth [26-28]. This then led to the question of tissue stem cells; whether or not they were subject to the Hayflick limit and whether they might possess telomerase activity. Anyone who trained with Don will immediately recognize the process I have just described, one in which Don would invariably take a yellow legal pad, a stack of which he always kept on hand, and proceed to fill page after page with written notes and diagrams littered with interesting ideas innumerable questions and potential experiments. Such sessions could last multiple hours, highlighting both the generosity with which Don freely gave of his time, as well as his seemingly limitless energy and enthusiasm. Despite the many responsibilities and demands on him, when you were with Don it was as though he had nothing else in the world to do, and that there was nowhere else he would rather be than with you discussing science and exchanging ideas. Don would also frequently write encouraging advice on the white board in the lunchroom, as well as more personalized notes which were left for us at our desks (**Figure 1**). Ultimately, we decided to use this in vivo experimental rodent system to explore the role of telomerase in stem cells of the prostate. At baseline, in the normal largely quiescent adult prostate, we found no evidence of telomerase activity. However, in stark contrast, we found high levels of telomerase in the residual involuted prostate gland following androgen ablation [29]. Since the cells within such an involuted gland are capable of fully regenerating the normal intact prostate gland, upon androgen restoration, from an operational perspective they must, at the very least, be enriched in prostate stem cells. Thus, we concluded that our results provided evidence for androgen-regulated telomerase activity in normal prostate stem cells. To our knowledge, this is the first ever demonstration of telomerase activity in stem cells. Upon testosterone restoration, this telomerase activity rapidly declined back to undetectable levels as stem cells became diluted out and returned to a quiescent, non-dividing state during the process of gland regeneration. Soon after, these results were confirmed by Ravindranath et al, in a non-human primate model [30]. In normal human prostate epithelial cells, the telomerase gene is suppressed by activated Androgen Receptor (AR) signaling, via AR binding to the telomerase gene promoter [31]. In sharp contrast, the activated AR induces telomerase expression in prostate cancer cells, and this effect is reversed upon androgen withdrawal. Thus, androgen regulation of telomerase expression in prostate

cancer cells is exactly the opposite of that observed in normal prostate epithelial cells [32-33]. In addition to abnormal AR signaling, the telomerase gene, as well as the gene for the telomerase RNA template subunit, are also positively regulated by the MYC oncogene, which, itself, is thought to be a major driver of prostate cancer [34-42].

Multidisciplinary research and telomere shortening in premalignant lesions

One of Don Coffey's signature quotes was, "When two minds come together a third mind is created". Don was a champion of the collaborative spirit in research. He explicitly led the charge for what we now call "multi-disciplinary team science", long before it became the catchphrase it is today. Don truly loved people and genuinely cared about and valued everyone who he came in contact with, whether they were technicians, students, postdocs, custodial staff members or cab drivers; Don would take time to find out about you -- your name, your family and personal histories and your thoughts and interests. When he asked you how you were, it was not just a formality - he really wanted to know how you were doing. In research, Don stressed that each person brought with them a unique set of knowledge, experience and talent that could be amplified in teams to synergistically address scientific problems. Of course, individuals also bring differing personalities, biases, preconceptions and egos. One of Don's special talents was to quickly create an egalitarian environment in group settings, where all felt equal and at ease. This spirit lives on his trainees, and I have endeavored to carry on this tradition as best as I can. After leaving Don's lab to seek a research faculty position for myself, I continued to focus on telomere biology in cancer. During my postdoc with Angelo De Marzo (yet another Don Coffey trainee) in the Johns Hopkins Department of Pathology, we were able to develop an in situ staining method allowing us to quantify telomere lengths at the single cell level in standard formalin-fixed paraffin embedded archival human tissue samples [43]. Using this technique, we were able to determine that dramatic telomere shortening is already evident in prostatic intraepithelial neoplasia lesions - widely considered to be initiated, pre-malignant, microscopic prostate cancer precursors [44]. This result, confirmed by others, implied that telomere loss is actually a very early event in prostate cancer development, likely contributing to tumorigenesis via the instigation of chromosomal instability due to critical telomere loss and telomere dysfunction [45, 46]. Don was very excited by this technique, because of the results we were able to obtain, but also because it produced dramatic multicolor images which appealed to his love of visual data. We quickly collaborated with several other specialists in a wide variety of cancers and found that, as in the prostate, the majority of precancerous lesions in most other organ systems also possessed abnormally short telomeres [47]. Next, we established a truly multidisciplinary collaborative team made up of prostate cancer pathologists, basic cancer researchers and cancer epidemiologists in order to assess the potential for telomere length measurement in patient samples to act as a useful biomarker for prostate cancer risk, prognosis, or for prediction of therapeutic response. From the outset, our team (several of whom were Coffey trainees) has worked together in true Don Coffey collaborative spirit, and this model has proven to be highly successful. Among various findings of the team to date, we have discovered associations between telomere abnormalities in diagnostic prostate biopsy specimens and risk for subsequent prostate cancer, between telomere lengths and cigarette smoking and obesity, and a strong association between telomere lengths in radical prostatectomy specimens and lethal outcome (disseminated metastasis or death from prostate cancer) [48-51]. We are currently exploring the potential ability of our telomere biomarker to predict patient benefit from added androgen ablative therapy in the setting of salvage radiotherapy post-prostatectomy for patients with rising PSA. This collaborative team exists in large part due to the influence that Don had on many of us during our formative years as young trainees, and has been published on as a model for successful team science [52].

White crows and extreme telomere shortening in non-malignant tissues

"If you see a white crow, that's telling you something". This was a phrase Don used to highlight the fact that, when you observe unusual data, you should pay special attention to it. "When something appears contrary to your hypothesis or expectations, that's when you'll learn something new". One example of such a white crow

was described above - the finding of abnormally short telomeres in premalignant lesions in a variety of human tissues (e.g. PIN in prostate, adenomas in the colon, DCIS in breast) [48]. From these results we learned that extreme telomere loss had already occurred by the earliest histologically recognizable stages of cancer, thus implicating telomere shortening as a potential causative factor, contributing to full malignant transformation rather than just being an epiphenomenon due to the high degree of cancer cell proliferation accumulated during tumor development. This particular phenomenon was most surprising in the case of testicular germ cell tumor precursors (ITGCNU lesions), which, similarly to epithelial cancer precursors also displayed short telomeres [53]. Unlike normal somatic cells, where telomerase expression is stringently suppressed, germ cells in the normal testis express high levels of telomerase expression, in order to maintain telomere lengths and guarantee transfer of a fully intact telomere complement to the next generation [54, 55]. Additional white crows have appeared from time to time. One that is particularly provocative is the discovery of significant telomere loss in histologically normal breast epithelial cells in females [57]. This finding appears to be quite prevalent among all women, and we hypothesize that it may be the result of hormonally-driven cyclic involution and re-growth of this cell population [56, 57]. This may help to explain why most breast cancers possess a luminal epithelial cell phenotype, and why breast cancer is so common, due to the fact that virtually all women harbor a sizeable pool of luminal epithelial cells primed to undergo genomic instability, should the normal cell senescence checkpoint (Hayflick limit) be breeched.

Conclusion

As I mentioned above, I feel incredibly blessed to have had the opportunity to know and work closely with Don Coffey over the past two decades. I hope that this brief glimpse into some of my experiences during my training under Don gives a glimpse of what this remarkable man was like, and that it will bring a knowing smile to those lucky individuals who also had the pleasure of sharing Don's company. Don was the finest human being I have yet had the pleasure of meeting. He was one of a kind; a national treasure, and will be sorely missed. I feel that without question, Don helped to make this world a far better place while he was with us. His spirit, kindness, love of life and his fellow human beings, and his endless enthusiastic curiosity were kindled in all of us who knew him. I will never forget Don, and will do my best to pass on his spirit.

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