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Review in Depth

FOXO3 and Related Transcription Factors in Development, Aging, and Exceptional Longevity

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In June 2013, a workshop was convened in San Francisco to explore, in depth, the role of the Forkhead transcription factor FOXO3 (and related FOXOs) in development, aging, and, in particular, exceptional longevity. The presentations covered results derived from model systems, computational analysis and bioinformatics, and genomics and genome-wide association studies of a number of cohorts. Although the data collectively strongly reinforce FOXO3 and the FOXO/FOXO3 pathway as very important determinants in aging and life span, much of the detail of how the latter is achieved still remains unknown, in part, because of the very large number of genes (~2,200 in *Caenorhabditis elegans*) the transcription factor is involved in helping regulate. Particularly challenging at the present time is understanding the association of apparently nonfunctional specific variants (single nucleotide polymorphisms) of FOXO3 and exceptional longevity in humans, a finding replicated in a number of studies. Nonetheless, as summarized in this report, valuable information and insights were presented at the workshop on the transcription factor including but not limited to its role in determining longevity in *C elegans* and *Drosophila* (in flies, eg, an important interaction in aging occurs between dFOXO and the transforming growth factor-β/activin pathway), stem cell function and aging (notably in hematopoiesis), downstream regulatory activity (eg, by binding near sites of RNAse occupancy and altering chromatin structure), and as a potential target for the development a healthy aging drug (in this example, using compounds developed and screened to effect FOXO function in cancer cells).

Key Words: FOXO3—Longevity—Aging—Transcription factor.

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FORKHEAD transcription factor (FOXO)/DAF-16 is a member of Forkhead transcription family that has been strongly implicated to play important roles in development, aging, and longevity. Much of this conclusion has been based on work done on model organisms, notably *Caenorhabditis elegans, Drosophila*, and mouse, but there is also compelling, if not conclusive, evidence linking one particular FOXO, FOXO3, to exceptional longevity in humans. However, many questions remain about how FOXO/FOXO3 work at a fundamental level, frustrating efforts to use this transcription factor as a basis for developing drugs designed to slow the aging process and improve healthy aging. This meeting brought together expert investigators working on FOXO/FOXO3 from different perspectives with the related goals of sharing state-of-the-art information on the functions and functional genomics of the transcription factor, fostering interdisciplinary dialogue and providing a format for discussing new research strategies and collaborations.

FOXO3 in Human Aging

A number of earlier publications had shown that particular single nucleotide polymorphisms (SNPs) of FOXO3 in humans appear at higher frequency in individuals who achieve exceptional longevity, for example, centenarian status (1–3). However, not only is it uncertain how these polymorphisms contribute, at a functional level, to long life, but there is also limited information about whether these SNPs are also associated with aging-related phenotypes that might logically be expected to contribute to healthy aging and/or extended life span, for example, by reducing the risk of heart disease and potentially life-threatening age-related disease. Brad Willcox and his colleagues of the Kuakini Hawaii Lifespan Study Research Group are currently testing the hypothesis that particular FOXO3 (exceptional longevity-associated) genotypes act to reduce cardiovascular disease risk, thereby increasing life span. Preliminary data were presented from a nested case-control study originally drawn from the Kuakini Honolulu Heart Program. The

study involved older American men of Japanese ancestry, most of whom were in their 70s at baseline, almost 20 years prior. On average, cases survived to age 98 and controls to age 78. The data show that those with the putative protective genotype (heterozygous and homozygous) had a later onset of and less prevalent coronary heart disease than those with the more common allele. Homozygotes (two protective alleles) had less prevalent coronary heart disease than heterozygotes (one protective allele). Also, men with the protective genotype tended to have more favorable overall cardiovascular risk profiles, by at least some accepted measures, for example, total cholesterol levels. Follow-up studies involving more individuals, including both genders and other ethnic backgrounds, are under way.

Greg Tranah and Dan Evans of the San Francisco Coordinating Center (California Pacific Medical Center, San Francisco) are pursuing an analysis strategy generally similar to the one being conducted by Willcox; in fact, some work is being done collaboratively. In this instance, FOXO3 SNPs are being tested for association with a number of aging-related phenotypes and disorders using data from a number of consortium-associated cohort studies. These measures will include, but are not limited to, insulin/glucose levels, body mass index/waist to hip ratio, serum lipid levels, coronary artery disease, and bone mineral density/osteoporosis. The expectation is that by identifying FOXO3 SNP associations with such phenotypes, it will help direct future functional genomic studies to those cellular mechanisms and molecular pathways responsible for the FOXO3-longevity relationship.

Despite the substantial focus of this workshop on a specific gene and its orthologs, compelling arguments can be made that exceptional longevity is not the result of variants of a single gene but rather the result of carrying a genotype that includes "longevity-favoring" alleles of a number of genes acting in concert. Paola Sebastiani, Harold Bae, and Tom Perls of Boston University presented evidence in support of this idea (4). They found in a genome-wide association study of approximately 800 centenarians that a combination of 281 SNPs, which alone did not reach statistical significance in being associated with longevity, collectively and accurately distinguished centenarians from controls, a finding consistent with results published in other studies (5). In addition, Sebastiani and colleagues using nonparametric linkage analysis of sib-pairs from 172 New England Centenarian Study families who survived beyond sex and birth year controls were able to identify regions in five chromosomes associated with exceptional longevity. Annotation of these regions, using genome-wide association study findings from two longevity studies in New England Centenarian Study centenarians, identified both novel and previously known candidate genes associated with an exceptional life span including adenosine deaminase, RNA-specific, B2 (nonfunctional [*ADARB2*]) and myelin transcription factor 1-like.

At this stage, everyone recognizes the major challenge of understanding the genetics and functional genomics of human aging and longevity. Consequently, Nir Barzilai (Albert Einstein College of Medicine, New York) made a valuable contribution to the workshop by providing criteria that SNPs of a particular gene should meet in order to be recognized as authentic marker of "authentic" longevity genes/polymorphisms. These criteria include, but are not limited to, replication of findings (of association) in several cohorts, evidence of the existence of nonsynonymous change in or near the SNP of interest, demonstrable change in the phenotype of cells in vitro, carrying the SNP, and the presence of intermediate age-related phenotypes in vivo. In addition, specifically in studies involving centenarians, it is important that appropriate controls be selected and that there be a parallel between the positive effects of longevity candidate SNP(s) on both aging phenotypes (eg, resistance to cardiovascular disease) and exceptional longevity. At this time, Barzilai noted that FOXO3 has met some but not all of these criteria.

FOXO-Related Transcription Factors in Aging in Animal Models

Much of the general acceptance of FOXO/FOXO3 as a longevity gene(s) comes work done with *C elegans* and *Drosophila*. Heidi Tissenbaum presented data on *C elegans* showing that the different isoforms of DAF-16 (the nematode ortholog of FOXO) cooperate to modulate different but related phenotypes including longevity via different gene expression patterns, upstream kinases, and target gene activation. A particular focus of attention was the DAF-16f isoform that appears to play a major role in regulating life span (6). Deciphering precisely how this occurs remains a major challenge. To further understand how DAF-16 regulates life span, genome-wide association studies (ChIP-chip) were performed resulting in the identification of approximately 2,200 targets of DAF-16 in the *C elegans* genome in loci associated with growth, development, reproduction, as well as longevity. Tissenbaum also described new software developed by her and her collaborators to look at the signaling networks downstream of DAF-16.

Drosophila melanogaster has also proved to be a very powerful tool for understanding the role of FOXO in aging. Marc Tatar's presentation focused on dFOXO regulation of life span via insulin–insulin-like growth factor-1 signaling. For example, among the transcriptional targets of dFOXO is DLIP6 (*Drosophila* Insulin-like peptide 6) expression in the fat body. Elevated DLIP6 represses secretion of DILP2 from the brain resulting in extended longevity. dFOXO also affects the transforming growth factor-β/activin signaling pathway (7). In muscle, this control of activin slows the age-related decline in muscle performance, helps maintain muscle protein homeostasis through the regulation of autophagy, and extends life span. Collectively, the

data document how insulin-like growth factor-1 signaling, through dFOXO, modulates aging by a combination of cell autonomous and noncell autonomous activities.

The Functional Genomics of FOXO3 in Humans

As compelling as are the findings from model systems, ultimately the appeal of FOXO3 as a focus of interest in human aging comes back to association and functional genomic studies using human biologic samples. The initial study that linked polymorphisms in FOXO3 to exceptional longevity came from Brad Willcox and his colleagues in Hawaii (1). As noted previously, related research activity by Willcox and colleagues continues (8), but it is still further enhanced by the efforts of Tim Donlon, a member of the Kuakini Hawaii Lifespan Study group and Director of the Kuakini Genetics Laboratory. In this instance, the effort is to identify additional polymorphisms in the transcription factor, with a particular focus on DNA changes predicted to have functional consequences (5). Thus, sequencing of 120 kb of FOXO3 in 95 individuals (age 95 years or older) yielded 125 SNPs of which four are predicted using "RegulomeDB" to significantly modify transcription factor binding to the *FOXO3* gene. These polymorphisms are currently being investigated using case-control and longitudinal study analysis to identify any age-related phenotypes with which they may be associated.

The Donlon effort (and a number of projects described previously) would not have been possible without the application of powerful bioinformatics tools such as RegulomeDB. Trina Norden-Krichmar and Nik Schork (The Scripps Research Institute) presented a brief summary of gene regulatory mechanisms particularly as related to FOXO3, and then an overview and some initial findings from many of the databases and tools currently available to investigators doing functional genomics. The latter include, but are certainly not limited to, providing detailed information on transcription factors, including prediction of transcription factor motifs and binding sites (eg, TRANSFAC) and binding data (eg, ChIPBase and ENCODE). In addition, databases and tools are available, which integrate analysis of variants that occur within FOXO3 and the surrounding regulatory regions. These tools include, in addition to the previously mentioned, HaploReg, Genevar, SG-Adviser, and SnpEff and can help determine the likely functional consequences of nucleotide substitutions, such as SNPs, in the *FOXO3* gene region. An example using two of these bioinformatic tools (Genevar and GTEx) to query the expression quantitative trait loci data for FOXO3, yielded three promising SNPs associated with the regulation of gene expression. Interestingly, it was noted that the two programs did not suggest the functionality of the same SNPs, highlighting the importance of comparing findings from multiple sources. The presentation concluded with a demonstration of the functional annotation of the known variants in the FOXO3 genomic region using the SG-Adviser software. The annotation of variants can be used to suggest how disruptions in genes and gene regulatory features may influence the FOXO3 pathway.

The presentation of Yousin Suh (Albert Einstein College of Medicine) combined elements of a number of the preceding talks by presenting data using a combination of state-of-the-art sequencing techniques, bioinformatic tools, and functional assays to (a) identify functional variants among all possible variants discovered in approximately 1,000 candidate genes of aging and (b) demonstrate that some of the genetic variation could be associated with individual differences in molecular endpoints such as gene expression or protein function (eg, ref. 9). Of particular relevance to this workshop, Suh and her colleagues sequenced the proximal promoter, exons, exon–intron junctions, untranslated regions of FOXO3 in 51 centenarians and 51 controls, and they identified several potentially functional variants with predicted regulatory roles. One such candidate was located in the 3′ untranslated region. The functionality of this variant was tested using several approaches including incorporating the untranslated region into a luciferase reporter assay, genome-wide expression quantitative trait loci analysis, and expression analysis in B-lymphoblastoid cell lines derived from carriers of longevity-associated FOXO3 genotypes. The data showed that the 3′ untranslated region variant is associated with a modest but significant decrease in FOXO3 expression levels both in vitro and in vivo. However, and what is of prime importance here, is that this appears to be only the second demonstration of a functional, phenotypic change at the cell level using a variant of human FOXO3 previously shown to be associated with exceptional longevity (10).

Function and Functional Genomics of FOXO3 in Model Systems

Of course, understanding more concretely how FOXO3 interacts with the genome is likely to prove pivotal to deciphering it's mode of action, particularly on a more global or system-wide scale. Boudewijn Burgering (University Medical Center, Utrecht) and his collaborators (notably Astrid Eijkelenboom) have now done extensive, elegant work, principally using the Chip-seq technique, not only to establish sites of FOXO3 binding in the human genome but also the association of such binding to sites of RNAse II occupancy and histone acetylation and methylation; the latter serving as markers of transcriptional activity and chromatin accessibility to transcriptional activation (11,12). Their data show that a significant part of FOXO3 activity is mediated through binding of the transcription factor to enhancers that can work at a distance from target genes, including chromatin loops which would further increase the genomic distance over which FOXO3 can act. The latter may represent the first demonstration linking FOXO3 activity to

chromatin structure; something that may vary between cells of different types and, therefore, provide a functional explanation for cell-type specific FoxO3a activity.

One fundamental biologic function where altered FOXO3 regulatory activity might reasonably be expected to have an effect on aging and longevity is stem cell activity. Three important articles dealing with this possibility were covered during the workshop. One of these studies, presented by Matthew Warr from Emmanuelle Passegué's Laboratory at UCSF, San Francisco, involved hematopoietic stem cells (HSCs) and focused on the role of FoxO3a in regulating a "protective" autophagy program that helps maintain the hematopoietic compartment during aging (13). Specifically, Warr and his collaborators showed, using GFP-LC3 mice and electron microscopy to track events, that blocking stress-induced autophagy by genetic or chemical means led to increased apoptosis and loss of survival of HSCs. Moreover, they found that FoxO3a was essential to maintaining a gene expression program that prepares HSCs for stress-induced autophagy, and that genetically suppressing FoxO3a reduced the autophagic response. Somewhat surprisingly, they also noted that "old" HSCs continue to maintain an intact FOXO3-driven autophagy system, and that this function is essential to the survival of "old" HSCs. They are currently testing the hypothesis that maintenance of autophagy into old age may contribute to the development of age-related diseases by allowing the survival of damaged, dysfunctional, or transformed "old" HSCs.

Still further support of the role of FOXO transcription factors in hematopoietic stem cell development and hematopoiesis was provided by Saghi Ghaffari (Mt. Sinai School of Medicine) as part of a comprehensive presentation that also included findings on FoxO1 function in hESC and SIRT-1 in regulating hematopoietic stem cell aging and lineage specification mediated by FOXO3 (e.g ref. 14). However, what were particularly striking were her results, which showed that in the absence of FOXO3 (in the mouse), reactive oxygen species-dependent DNA damage was increased in HSC (notably in primitive LSK cells) and that the ability of stem cells to competitively repopulate the marrow of irradiated animals was diminished. Moreover, red cell development was also significantly compromised; specifically, the enucleation process was defective in the absence of FOXO3, and mitophagy (selective mitochondrial autophagy) was decreased. The latter results reinforce the connection between FOXO3 and autophagy (see Warr previously described) and emphasize the role of the latter in maintaining the functionality of cells at all stages of hematopoiesis.

Blood cell formation is not the only circumstance in mice where FOXO3 plays a critical role in stem cell maintenance. As reported by Renault and colleagues from Stanford, loss of this transcription factor in FoxO3 null mice depletes neural stem cells in vivo (in both the subventricular zone and hippocampus of the brain) and impairs the ability of the latter to self-replicate in vitro (15). Precisely what genes and genetic pathways essential to neurogenesis are affected by the absence of FOXO3 are not known, but Chip-seq experiments performed by Webb and colleagues indicate some 2,200 target genes have binding sites for the transcription factor. The latter target group is enriched for genes associated with longevity and/or with neurodegenerative disease. The Chip-seq experiments also revealed that a high percentage of the genes bound by FOXO3 are also targeted by the pro-neurogenic transcription factor Ascl1. Analysis of the functional relationship between the two transcription factors indicate that FOXO3 blocks the Ascl1-mediated induction of neurogenesis from neural stem cells and hinders the reprogramming of fibroblasts into neural cells. The antagonistic relationship between the two transcription factors suggests that they act together to balance neural stem cell self-renewal and nerve cell differentiation in the adult (mouse) brain (16).

Translational (Drug Development) Efforts

Of course, in the final analysis, the goal of much of this research effort is to develop pharmaceuticals that will slow the aging process and, hopefully by doing so, extend the period of healthy aging. To the best of our knowledge, to date, no drug development efforts have been undertaken using FOXO3 as a focus of activity, with slow/healthy aging the intended and hoped for outcome. However, there has been significant research using this approach for the purpose of developing anticancer drugs. One investigator prominent in the latter effort is Wolfgang Link (Universidade do Algarve, Portugal), who has developed high-throughput, screening assays systems to monitor FOXO3 protein cytoplasm–nuclear translocation and subsequent downstream transcription factor activity (17). Using these outcome measures to screen multiple small hairpin RNAs, compounds of known activity, and approximately 35 k structurally diverse small molecules, Link and his colleagues discovered FOXO-repressor functions associated with several human genes. They also identified a group of 200+ chemical agents capable of inducing FOXO translocation into the nucleus, with a subset that directly suppressed PI3Ka, an upstream inhibitor of FOXO activity. From the results of the nuclear translocation assays, a structure-activity profile was developed from pyrazolopyrimidine derivatives that ultimately provided computational and experimental data, which showed that the R1 and R4 positions on pyrazolopyrimidine were the preferred sites for morpholine and meta-phenol substitutions. These experiments lead to the synthesis of a pyrazolopyrimidine (ETP-45658) that effectively and specifically inhibits PI3Ka. ETP-45658 has now been shown to inhibit several PI3Ka pathway active in tumor cell lines, to inhibit the growth of five cancer cell lines and, consistent with the

latter, alter the expression of FOXO target genes related to the cell cycle. Promisingly, the mechanism of action of ETP-45658 has been confirmed in mammary ductal cells in vivo. (Note: One tangible consequence of the workshop is a new collaboration between Link and Gordon Lithgow [Buck Research Institute for Research on Aging, Novato, CA] to systematically test some of the compounds described previously in *C elegans* for affects on the aging process and longevity.)

Conclusion

In all, the FOXO/FOXO3 workshop met its intended objectives perhaps, the most important one being, to provide a supportive environment for concentrated investigator-to-investigator interaction. For the participants, this contributed to both a broader and deeper understanding of the possible roles of FOXO/FOXO3 in aging (as seen from multiple perspectives) and the beginnings of new interdisciplinary collaborations. It also clearly identified important gaps in knowledge and the major challenges that remain, particularly if the hope is to use understanding of this transcription factor, and the genes it helps control, to develop therapeutics that will improve healthy aging and increase health span.

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