Genetic and Genomic Approaches to Understanding Macrophage Identity and Function

Christopher K. Glass

Abstract—A major goal of our laboratory is to understand the molecular mechanisms that underlie the development and functions of diverse macrophage phenotypes in health and disease. Recent studies using genetic and genomic approaches suggest a relatively simple model of collaborative and hierarchical interactions between lineage-determining and signal-dependent transcription factors that enable selection and activation of transcriptional enhancers that specify macrophage identity and function. In addition, we have found that it is possible to use natural genetic variation as a powerful tool for advancing our understanding of how the macrophage deciphers the information encoded by the genome to attain specific phenotypes in a context-dependent manner. Here, I will describe our recent efforts to extend genetic and genomic approaches to investigate the roles of distinct tissue environments in determining the phenotypes of different resident populations of macrophages. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.304051.)

Key Words: genetics ■ genomics ■ macrophages ■ transcription factors

Preface

It is a great honor to give this year’s Russell Ross Memorial Lectureship in Vascular Biology. Dr Ross made many seminal discoveries in this field, trained a generation of leading investigators, and was one of the major proponents of the concept that atherosclerosis is an inflammatory disease. I initially met Dr Ross at a Gordon Research Conference on Atherosclerosis that was the very first scientific meeting I attended. My graduate work at that time was focused on lipid metabolism and the idea that atherosclerosis was a lipid disease. The opportunity to discuss my work with Dr Ross and to get his perspective at this meeting and during subsequent encounters had a major influence on my ultimate scientific directions. A long-standing interest of my laboratory has been to integrate the inflammation and lipid theories of atherosclerosis by studying the intersection of lipid metabolism and macrophage gene expression. In this lecture, I will present recent studies that attempt to advance our understanding of how tissue environment drives the selection and function of enhancers that control tissue-specific macrophage identities.

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Introduction

Macrophages reside in essentially all tissues of the body and play key roles as sentinels of infection and injury. In addition, each population of macrophages within a tissue takes on specialized functions that are tuned to the developmental and functional requirements of that tissue. For example, microglia, representing the main population of macrophages within the nervous system, play roles in phagocytosis of apoptotic neurons and synaptic pruning. In the spleen, macrophages phagocytose senescent red blood cells and participate in iron recycling. Even within a single tissue, macrophages can exhibit heterogeneous phenotypes. Distinct populations of macrophages resident in the peritoneal cavity can be distinguished on the basis of morphological criteria and different levels of major histocompatibility complex class II expression. Although macrophage heterogeneity is normally tuned to support normal tissue homeostasis, the ability of these cells to acquire distinct phenotypes in response to their environment can result in pathogenic consequences. This scenario is exemplified by a diversity of macrophage phenotypes within atherosclerotic lesions as defined by variation in lipid accumulation and distinct surface markers. Although most macrophages within the artery wall are thought to promote lesion development, some may be protective.

These observations raise the question of how distinct populations of macrophages are established and the extent to which different tissue environments play instructive roles with respect to their phenotypes. The recent development of genomic approaches that are based on the ability to sequence millions of short DNA fragments has revolutionized the approach to this type of question. It is now possible to globally quantify the broad spectrum of RNAs that are produced by a cell or tissue (mRNAs, miRNAs, etc) by converting these RNAs to libraries of DNA copies that can be deeply sequenced (referred to as RNA-Seq). In addition, it is also possible to globally define the genomic locations of specific histone modifications and transcription factors of interest.

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using chromatin immunoprecipitation linked to deep sequencing (referred to as chromatin immunoprecipitation sequencing (ChIP-Seq)). In this method, cells are treated with a cross-linking agent to covalently link transcription factors and histones to DNA. The DNA is then sheared into small fragments and subjected to immunoprecipitation with antibodies to the histone modification or transcription factor of interest. The crosslinks are subsequently reversed, and the purified DNA fragments are subjected to deep sequencing. The sequenced tags are then mapped to the genome. Tag accumulations at specific regions of the genome indicate that the marked histone or transcription factor of interest was present, with the overall pattern providing a genome-wide histogram of their locations. By combining ChIP-Seq and RNA-Seq approaches, it has been possible to investigate mechanism by which transcription factors drive cell-specific patterns of gene expression on a global scale.

**Environment Is a Major Determinant of Resident Macrophage Gene Expression**

As a starting point for investigating the influence of environment on macrophage gene expression, we performed RNA-Seq analysis of 3 populations of resident macrophages: microglia, large peritoneal macrophages (LPMs), and small PMs (SPMs).5,6 LPMs and SPMs share many features of macrophages, including expression of the colony-stimulating factor 1 receptor, F4/80, and MerTK, but they can be distinguished by low (LPM) or intermediate (SPM) expression of major histocompatibility complex II. These resident macrophage populations were chosen for 2 reasons. First, they permitted an analysis of macrophages residing in different environments (eg, LPMs and microglia) and different macrophages in the same environment (LPMs and SPMs). Second, we were successful in developing methods of isolation that provided sufficient cells for genome-wide analysis and also preserved their in vivo gene expression and histone modification profiles.

RNA-Seq analysis indicated striking differences between LPMs and microglia, with ≥7000 mRNAs exhibiting significant differences in expression. Taking a stringent threshold of a >16-fold difference, >500 mRNAs were preferentially expressed in microglia, whereas >600 mRNAs were preferentially expressed in LPMs. In contrast, LPMs and SPMs exhibited a much more similar pattern of expression, with ≥800 mRNAs exhibiting significant differences. A total of 108 genes were expressed at >16-fold higher levels in SPMs, whereas only 5 mRNAs were expressed at >16-fold higher levels in LPMs. We compared these results with RNA-Seq data obtained for thioglycollate-elicited macrophages and bone marrow–derived macrophages, which are 2 widely used macrophage model systems. Clustering analysis indicated that SPMs and LPMs were closely related and thyoglycollate-elicited macrophages and bone marrow–derived macrophages were closely related. In addition, these 4 cell types were much more similar to each other than they were to microglia. We found that each type of macrophage was also distinguished by a unique gene expression pattern that was consistent with previous findings. For example, Cx3cr1 was much more highly expressed in microglia than any of the other 4 macrophage populations, whereas PMs preferentially expressed Gata6. SPMs expressed much higher levels of the Ciita mRNA that encodes a transcription factor necessary for expression of major histocompatibility complex class II gene expression. Overall, the much greater differences in gene expression between LPMs and microglia in comparison with LPMs and SPMs suggest an important role of environment in determining the subset-specific patterns of gene expression.

To directly examine the influence of environment on microglia and LPM gene expression, we placed each cell type into culture for 7 days in the presence of macrophage colony-stimulating factor or interleukin-34, factors that induce signaling through the colony-stimulating factor 1 receptor and maintain macrophage survival. Using RNA-Seq to measure mRNA levels, we observed striking changes in gene expression in both cell types in comparison with the patterns observed in vivo. A large fraction of the genes exhibiting preferential expression in LPMs versus microglia were significantly downregulated in LPMs in culture. Similarly, a large fraction of the genes exhibiting preferential expression in microglia versus LPMs were significantly downregulated in microglia in culture. Overall, each cell type preferentially lost expression of genes that represented the molecular signature of that cell type in vivo, indicating that the identities of microglia and LPMs require constant environmental input.

**Environment Activates Common and Subset-Specific Enhancers**

These findings raise the general question of how different macrophage identities are established and maintained. Gene expression is regulated at many levels, but gene transcription represents an essential and, in many cases, dominant point of control. Protein-coding genes are transcribed from promoters, which represent genomic regions that recruit basal transcription factors and RNA polymerase II. Physiological levels of gene expression and responses to internal and external signals require the actions of additional sequence-specific transcription factors that recruit nucleosome-remodeling complexes, histone-modifying proteins, and other factors to regulate polymerase-II activity. Such factors can bind in close proximity to promoters to influence gene expression. However, there is substantial evidence that additional genetic elements referred to as enhancers play major roles in determining cell-specific patterns of gene expression. Initially identified >30 years ago, enhancer elements can be located at various distances from promoters, typically between 1 and 50 kilobases away, and rarely as far as 500 kilobases distant. They can also be positioned upstream, downstream, or within the genes they regulate. Like promoters, enhancers are occupied by sequence-specific transcription factors that recruit nucleosome-remodeling factors and histone-modifying proteins. To a much greater extent than promoters, enhancers are occupied by lineage-determining transcription factors (LDTFs) that are required for the development of specific cell types. They also exhibit a distinct histone modification signature in which histone H3 lysine 4 exhibits more monomethylation (H3K4me1) or dimethylation (H3K4me2) than trimethylation
(H3K4me3), which is a mark of promoters (Figure 1A and 1B). Remarkably, annotation of the human genome for histone modifications and other features of enhancers in dozens of different cell types suggest the existence of ≈1 million enhancer elements, many more such elements than genes. These studies further suggest that each cell type selects a subset of ≈30,000 to 50,000 enhancers from this vast set of enhancers that in turn serve as the genetic regulatory elements specifying the identity and regulatory potential of cells.

A major effort of our laboratory has been to investigate mechanisms by which macrophages select their specific complements of enhancers from the genome. We gained important insights into this question by studying the genomic binding sites of PU.1, a LTDF that is required for the development of both macrophages and B cells. Using ChIP-Seq, we found that PU.1 bound to different regions of the genome in macrophages and B cells that were associated with macrophage or B-cell–specific programs of gene expression, respectively. The basis for cell-specific binding was shown to result from collaborative interactions between PU.1 and alternative transcription factors that were differentially expressed in each cell type. CCAAT-enhancer-binding protein factors were found to be important collaborators with PU.1 in macrophages. Regions of the genome containing closely spaced recognition motifs for PU.1 and CCAAT-enhancer-binding proteins became occupied by combinations of these factors in macrophages, whereas PU.1 did not bind to these regions in B cells. Conversely, EBF and Oct factors were important collaborators with PU.1 in B cells (Figure 2). Regions of the genome containing closely spaced recognition motifs for PU.1 and Oct or EBF became occupied by these factors in B cells, but they were not occupied by PU.1 in macrophages. Thus, different combinations of factors functioned to prime distinct genomic regions for enhancer activity. Importantly, these primed regions of the genome were the major binding sites for signal-dependent transcription factors (SDTFs), such as nuclear factor-κB and nuclear receptors. These SDTFs in turn recruited additional factors, including histone acetyltransferases, that transformed poised enhancers to active enhancers. Thus, even though SDTFs can be broadly expressed and can respond to similar signals, they can direct different transcriptional responses because they are directed to cell-specific enhancers. A similar hierarchical relationship of LTDFs and SDTFs was found in regulatory T cells, embryonic stem cells, and dendritic cells.

Observations made through the comparison of macrophages and B cells led us to wonder whether similar mechanisms would underlie the distinct transcriptional programs of resident PMs and microglia (Figure 3A). To address this question, we initially performed ChIP-Seq experiments for H3K4me2, histone H3 acetylated at lysine 20 (H3K27ac), and PU.1 in LPMs, SPMs, and microglia. H3K4me2 marks both enhancers and promoters and is present at both primed and active regulatory elements, whereas H3K27ac is a mark associated with active promoters and enhancers. We found that similar to patterns of gene expression, microglia, and LPMs exhibited a much more different pattern of H3K4me2-marked regions than observed for the comparison of LPMs and SPMs. The differences observed for microglia and LPMs were predominantly at enhancer-like regions. Of 8439 promoters, only 329 showed >4-fold differences between cell types. In contrast, of ≈50,000 distal regions marked by H3K4me2, 12,500 showed >4-fold differences. Overlaying
H3K27ac data on regions marked by H3K4me2 allowed estimation of the extent to which both common and cell-specific enhancers were active. We found evidence that in addition to activation of microglia-specific or LPM-specific enhancers, many enhancers that were primed in all 3 cell types (based on H3K4me2) were only activate in 1 or 2 cell types (based on H3K27ac). For example, an enhancer adjacent to the Rarb gene, encoding a retinoic acid (RA) receptor (RAR) and also a RA-responsive gene, exhibited H3K4me2 in microglia, LPMs, and SPMs, but it was almost exclusively marked by H3K27ac in LPMs and SPMs. This is significant from the standpoint of understanding the effect of environment on gene expression, because omentum-derived RA has recently been found to be an important inducer of the LPM phenotype by stimulating RAR-dependent transcription.5 Our findings are consistent with RA being an important environmental factor in the peritoneum, but not in the brain, and acting on a common poised enhancer to selectively induce Rarb expression in the peritoneal population of macrophages.

The finding that microglia and LPMs exhibit activation of both common and distinct enhancers led us to consider the possibility that, like differences between macrophages and B cells, PU.1 would collaborate with alternative sets of transcription factors to prime subset-specific enhancers (Figure 3A). We therefore performed ChiP-Seq for PU.1 in microglia and large PMs. In addition to binding to a common set of genomic locations, we found that PU.1 localized to thousands of different regions of the genome in each macrophage subset.2 We performed de novo motif analysis of the microglia-specific and LPM-specific binding sites to identify transcription factor recognition motifs colocalizing with PU.1 at these locations. As expected, an identical consensus PU.1 motif was found at both microglia-specific and LPM-specific binding sites, analogous to our earlier findings at macrophage and B-cell–specific binding sites. In contrast, the other sequence motifs colocalizing with PU.1 binding were completely different in microglia and LPMs. Recognition elements for CCAAT-enhancer-binding proteins, activator protein-1 factors, GATA factors, and RARs were highly enriched near PU.1 binding sites in LPMs, whereas recognition motifs for HIC3, Mef2, unknown factor, and Smad proteins were coenriched near microglia-specific PU.1 binding sites. These findings are of significance because of the recent discovery of the importance of RA and GATA6 in the development of LPMs5,26,27 and the recently established requirement of brain-derived transforming growth factor-β (TGF-β), which regulates gene expression through SMAD transcription factors, in microglia development.28,29 Collectively, the factors recognizing motifs coenriched with PU.1 are putative LDTFs or SDTFs that serve to drive LPM-specific or microglia-specific enhancer selection and activity.

**Natural Genetic Variation Enables Discovery of Collaborating Transcription Factors**

To further investigate the mechanisms establishing microglia and LPM-specific enhancers, we exploited the natural genetic variation provided by inbred laboratory and wild-derived strains of mice on the binding of PU.1 in each cell type. Because binding of PU.1 to chromatin requires collaborative interactions with other transcription factors, single-nucleotide polymorphisms can disrupt PU.1 binding not only by occurring in the PU.1 recognition motif but also by disrupting the recognition motif for a nearby collaborative factor30 (Figure 3B). Therefore, if PU.1 binding is lost at a genomic region at which there is no alteration in the PU.1 binding site, the loss of binding is likely because of a mutation in the recognition motif for a collaborative factor. Regions of the genome exhibiting strain-specific binding of PU.1 can be...
systematically interrogated for such mutations. The ability of this approach to identify motifs for collaborative transcription factors is dependent on the number of informative events of strain-specific binding of PU.1 at locations at which the PU.1 recognition motif is intact.

To explore the feasibility of this approach, we performed ChIP-Seq for PU.1 in microglia and LPMs derived from nonobese diabetic mice and SPRET mice. Compared with the C57BL/6J reference strain, nonobese diabetic mice exhibit ≈5 million single-nucleotide polymorphisms, whereas SPRET mice exhibit ≈40 million single-nucleotide polymorphisms.31 We found thousands of strain-specific PU.1 binding sites in each cell type at which the PU.1 site was not mutated, providing a large number of informative events for analysis. We systematically evaluated these genomic regions for mutations in the recognition motifs for the 100 most highly expressed transcription factors in microglia or LPMs. Of these, mutations in >20 of these motifs were found to be significantly associated with strain-specific binding of PU.1.9 Importantly, this analysis recovered recognition motifs for CCAAT-enhancer-binding proteins and activator protein-1 factors, which we previously established as collaborative factors with PU.1 through gain and loss of expression studies (Figure 3C). In addition, we identified a dozen motifs that were associated with LPM-specific binding of PU.1 and 4 motifs associated with microglia-specific binding. Motifs associated with LPM-specific binding included recognition elements for GATA factors, KLF4, and MEF2c, whereas motifs associated with microglia-specific binding of PU.1 included recognition motifs for MAFb and SMAD3 (Figure 3C). These findings therefore confirmed the results of motif enrichment analysis and identified motifs for factors not previously known to contribute to macrophage development.

With these findings in hand, we then revisited the question of how environment controls LPM- and microglia-specific gene expression. Recent studies of LPMs indicate that their maturation and function are dependent on omentum-derived RA that is an activating ligand for RARβ. RARβ in turn induces the expression of GATA6.3 Conversely, TGF-β signaling in the brain is essential for maintenance of microglia.28,29 Although an important role of RARβ has been established, we found that mRNAs encoding all 3 nuclear receptors for RA (Rara, Rarb, and Rarg) are highly expressed in LPMs compared with microglia, whereas the main receptors for TGF-β signaling (Tgfbr1 and Tgfbr2) are preferentially expressed in microglia compared with LPMs. Remarkably, all 3 Rar genes were found to be significantly downregulated when LPMs were transferred to culture conditions for 7 days. In addition, nearly 2 thirds of the transcription factors recognizing sequence motifs identified by analyzing effects of genetic variation were also downregulated under these conditions, including GATA6.5

We then treated LPMs in culture with RA for 7 days to determine the extent to which the in vivo LPM phenotype could be maintained. Notably, expression of approximately half of the genes that exhibit LPM-specific expression compared with microglia was maintained by RA treatment. In contrast, <5% of the genes exhibiting common expression between LPMs and microglia were RA-dependent. Although Rarb expression was maintained by RA treatment, expression of Rara and Rarb was not, indicating that environmental factors other than RA are required. Similarly, of the additional collaborative factors identified by analysis of effects of genetic variation, Gata6, Bhlhe40, and Tfeb were maintained by RA treatment, whereas the remaining environment-dependent factors were not. Conversely, we also treated these LPMs in vitro with TGF-β to determine the extent to which their gene expression program could be converted to that of microglia. TGF-β induced expression of about half of the genes that are normally highly specific for microglia, but only 4% of the genes that are commonly expressed between these cell types. Therefore, RA and TGF-β primarily regulate genes that specify LPM and microglia phenotypes, respectively.8

Effects of removal from the in vivo environment and treatment with RA or TGF-β on gene expression were also mirrored at the level of enhancers. Removal of LPMs from the peritoneal cavity resulted in the loss of H3K4me2/H3K27ac
Enzymes account for part of the different patterns of gene expression observed in LPMs and microglia. Importantly, however, our findings also indicate that actions of environmental factors on common poised enhancers lead to the differential expression of alternative transcription factors that collaborate with PU.1 to select subset-specific enhancers. Thus, environment drives tissue-specific programs of gene expression through both direct and indirect activation of subset-specific enhancers.

We speculate that this hierarchical mechanism of enhancer selection and activation operates in other macrophage subsets and probably other tissue-specific cell types. The discoveries of TGF-β and RA as key regulators of in vivo macrophage phenotypes resulted from the loss of function studies, but these findings also independently emerged from the analysis of tissue macrophage enhancers. We speculate that this approach could be used to identify other classes of signaling molecules, including lipids, in other tissue environments. The strong influence of environment on enhancer landscapes and gene expression suggests that the diverse phenotypes of macrophages observed within atherosclerotic lesions reflect, at least in part, substantial differences in the microenvironment.6

There are many factors that are present in lesions that affect macrophage phenotypes when tested in vitro, including oxidized lipoproteins, cytokines, cholesterol crystals, apoptotic cells, etc. Although the present studies were performed using hundreds of thousands of cells to obtain robust ChIP-Seq data sets, recent innovations in genomic technologies now enable these types of experiments to be performed with tens of thousands of cells or less.22,23 It is thus now possible to begin to interrogate many populations of interest within in vivo environments, including the artery wall, and to ask not only how tissue-specific enhancer landscapes are established but also effects of pathogenic stimuli and therapeutic interventions. It is worth noting here that emerging classes of small molecules that act on proteins that write, read, and erase histone modifications, such as histone deacetylase inhibitors and acetylated histone tail mimetics, exert many of their effects at enhancers.20,34 Because each cell type selects a different complement of enhancers from the genome to establish its identity and regulatory potential, each cell type will also respond in a unique way to drugs that alter enhancer function. Recent genetic and pharmacological studies suggest that these interventions may have promise in the prevention and treatment of cardiovascular disease.35,36

Conclusions and Future Directions

Our findings suggest a hierarchical model by which LDTFs and SDTFS regulate macrophage identity and tissue-specific phenotypes5,10,30 (Figure 4). We propose that a core set of LDTFs, exemplified by PU.1, play essential roles in priming an enhancer landscape that is common to many types of tissue macrophages. Tissue-specific signals activate different sets of primed enhancers to direct different programs of gene expression. For example, although the enhancers in the vicinity of Rarb are primed in all macrophage subsets, they are only activated in the peritoneal cavity in response to local RA. Similarly, enhancers in the vicinity of Cx3cr1 are primed in all macrophage subsets, but these enhancers are selectively activated in microglia in response to local TGF-β. Direct actions of these signaling molecules on common poised enhancers account for part of the different patterns of gene expression observed in LPMs and microglia. Importantly, however, our findings also indicate that actions of environmental factors on common poised enhancers lead to the differential expression of alternative transcription factors that collaborate with PU.1 to select subset-specific enhancers. Thus, environment drives tissue-specific programs of gene expression through both direct and indirect activation of subset-specific enhancers.

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Disclosures

None.

References


Why did you choose the profession of scientific investigation?

Sometime during my Junior year at the University of California, Berkeley, I settled on the idea of pursuing clinical medicine as a profession. To get a better feeling the role of science in medicine, I started working in Alex Nichols’s lab in the Donner laboratory. This was where John Goffman had used the analytical ultracentrifuge to characterize low- and high-density lipoproteins and their relationship to heart disease. I found experimental work challenging and exciting and spent a lot of time in the lab. After my first year of medical school at the University of California, San Diego, I spent the summer working in John Kane’s laboratory in the Cardiovascular Research Institute at UC San Francisco. In addition to virtually daily contact with John, I got to know many other great scientists at the CVRI, including Dick Havel, who was the director at the time. I didn’t accomplish much of significance in the lab despite my best efforts, but was so completely taken over by the idea of being a physician scientist that I applied for and was accepted into the Medical Scientist Training Program when I returned to UC San Diego in the fall. I have pretty much been on that track ever since.

Who have been your role model(s) in your scientific and professional life?

My PhD thesis advisor, Dan Steinberg, has been a central role model throughout my professional life and is the person who taught me that mentoring doesn’t stop when trainees leave the lab. John Kane and Joe Witztum were also great role models in the field of atherosclerosis research. Michael (Geoff) Rosenfeld, my post doc advisor and the person who trained me as a molecular biologist, has also been a lifelong role model for how to creatively approach important and challenging problems. Siamon Gordon was a great role model for how to study macrophage biology. There are many others.

If you were not a scientist, which profession would you pick?

A swim coach. I started at Cal on a partial swimming scholarship. After two years it became pretty clear that I wasn’t as good a swimmer as I hoped I would be, and I wasn’t getting a good return on the investment of a lot of time in the pool. I traded that in for the time in Alex Nichols’s lab and a more serious approach to my coursework. Had I been a better swimmer and competed for the full four years, it is unlikely that I would have made my way to medical school and much more likely into a coaching. I think I would have enjoyed the mentoring and scientific aspects of coaching a lot and still think about it as a volunteer activity down the road.

Which direction do you envisage your science taking?

I come in to the laboratory every morning with the idea that the best papers from my lab have yet to be written. Right now we are planning to apply genomic approaches to understand macrophage development and function in vivo. We hope to learn about how macrophages participate in both normal tissue homeostasis and pathological forms of inflammation in diseases ranging from atherosclerosis to neurodegenerative diseases.

What are your nonscientific activities?

Keeping up with our four kids (now grown). I still like to swim and am in the pool or the ocean most mornings before work. I greatly enjoy eating my wife’s cooking, and we like traveling together to new places. I don’t watch much TV.

What are you favorite foods and are they heart healthy?

I like lots of different foods that span the spectrum of today’s view of what is heart healthy. Having taught nutrition at one point to medical students, I came to realize that this is a moving target. I am interested in the compositions of foods beyond their content of essential sources of calories, building blocks, and vitamins. For example, there is a vast array of small molecules present in plants that have biological activities, but for most, we have very little understanding of their effects on tissue homeostasis and function. My goal is to adjust relative proportions of various foods to try to achieve a “high-performance diet.” Right now I am experimenting with fruit/vegetable juice extracts supplemented with chia seeds as a preworkout tonic.
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