Pattern Specification and Immune Response Transcriptional Signatures of Pericardial and Subcutaneous Adipose Tissue

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Abstract

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality in the United States. Recent studies suggest that pericardial adipose tissue (PCAT) secretes inflammatory factors that contribute to the development of CVD. To better characterize the role of PCAT in the pathogenesis of disease, we performed a large-scale unbiased analysis of the transcriptional differences between PCAT and subcutaneous adipose tissue, analysing 53 microarrays across 19 individuals. As it was unknown whether PCAT-secreted factors are produced by adipocytes or cells in the supporting stromal fraction, we also sought to identify differentially expressed genes in isolated pericardial adipocytes vs. isolated subcutaneous adipocytes. Using microarray analysis, we found that: 1) pericardial adipose tissue and isolated pericardial adipocytes both overexpress atherosclerosis-promoting chemokines and 2) pericardial and subcutaneous fat depots, as well as isolated pericardial adipocytes and subcutaneous adipocytes, express specific patterns of homeobox genes. In contrast, a core set of lipid processing genes showed no significant overlap with differentially expressed transcripts. These depot-specific homeobox signatures and transcriptional profiles strongly suggest different functional roles for the pericardial and subcutaneous adipose depots. Further characterization of these inter-depot differences should be a research priority.

Introduction

Cardiovascular disease (CVD) has long been the leading cause of death in the U.S. and the developed world [1]. While efforts to decrease the impact of the major cardiovascular risk factors have yielded modest success, there are indications that CVD mortality rates may rise again because of the epidemic of obesity and obesity-related comorbidities such as diabetes, hypercholesterolemia, and hypertension [2][3][4].

The relationship between adipose tissue and disease remains a very active area of study, and efforts have further intensified since the discovery that adipose tissue is an active, multifunctional endocrine organ [5][6]. In part because of the strong correlation between the size of the visceral adipose tissue (VAT) depot and the incidence of CVD, type 2 diabetes mellitus, and metabolic syndrome, attention has focused on understanding the molecular and functional differences between VAT and subcutaneous adipose tissue (SQAT) [7][8][9]. Compared to SQAT, human VAT secretes more interleukin-6 (IL-6), expresses more peroxisome proliferator-activated receptor gamma (PPARγ), and produces less adiponectin [10][11][12]. With regards to CVD, IL-6 has been particularly interesting because of its close association to inflammation, obesity, and coronary heart disease [13]. It is also a strong, independent marker for increased mortality in the setting of CAD [14].

Given the depot-specific functions seen in VAT and SQAT, we hypothesized that pericardial adipose tissue (PCAT) may play a distinct physiologic role. Anatomically, PCAT lies between the visceral and parietal pericardium and is therefore distinct from epicardial adipose tissue (EAT) [15]. Using data from the Framingham Heart Study, radiographic studies positively correlated PCAT volume with the incidence of coronary artery calcification, as well as the elevation of several metabolic risk factors [16][17]. Similar results were found using data from several other patient populations [18][19][20][21]. These studies, while well-designed, provide neither transcriptional nor protein-level insight into the physiology of PCAT. To our knowledge, we are unaware of any studies that did so. In contrast, EAT has been shown to both overexpress and oversecrete inflammatory markers [22][23][24][25].

Given the absence of non-radiographic data regarding the physiology of PCAT, we designed our study to identify transcriptional differences between PCAT and SQAT, and also between isolated pericardial adipocytes (pcAds) and isolated...
subcutaneous adipocytes (sqAds). Our study revealed that these depots express specific patterns of homeobox genes. We further found that PCAT and pcAds both overexpress atherosclerosis-promoting chemokines. These depot-specific homeobox signatures and transcriptional profiles strongly suggest different functional roles for the pericardial and subcutaneous adipose depots.

**Results**

Between June 2009 and March 2010, 19 patients undergoing elective cardiac operations at Massachusetts General Hospital were enrolled in this study. From these patients, RNA samples were isolated from SQAT, PCAT, sqAds, and pcAds, of which 53 samples (11 SQAT, 11 PCAT, 15 sqAds, and 16 pcAds) passed quality standards for hybridization to Affymetrix U133A Plus 2.0 microarrays. Using nuclear and lipid stains, we determined that our isolated adipocytes contained 96.7% single-nuclei adipocytes (Fig. S1).

As our primary aim was the identification of depot-specific transcription patterns, we first performed unbiased hierarchical clustering of whole tissue and isolated adipocytes. This showed clustering of samples which were processed in one specific month, suggesting a strong batch effect (Fig. S2). To remove this effect, principal component analysis was performed. After correction for batch effects in processing (see Methods) clustering analysis revealed 3 distinct clusters. One cluster was composed solely of pcAds samples, a second cluster contained 10 samples of which 9 were pcds, and a third cluster of 12 samples contained 9 pcAds samples (Fig. 1B).

We then used linear models to identify differentially expressed genes between populations PCAT and SQAT, and between pcAds and sqAds. Given the multiple hypothesis testing burden of microarray analyses, a nominal p-value of 0.05 almost certainly includes a large number of false positives. We thus restricted our analysis to those genes that were differentially expressed at a false-discovery rate (FDR) of <0.25 (Fig. 1C, Methods). In PCAT vs. SQAT, this approach identified 2,284 differentially expressed probesets (Fig. 1C). In pcAds vs. sqAds, this analysis yielded 657 differentially expressed probesets.

To characterize the differentially expressed transcripts, we used unbiased gene enrichment analysis (GEA) to look for enriched Gene Ontology (GO) terms in the candidate probesets overexpressed in PCAT, SQAT pcAds and sqAds individually. Since multiple probesets map to the same genes, a list of unique candidate genes were generated from probeset lists. These candidate genes were ranked in order of greatest fold-change and analyzed for GO category enrichment as ordered lists using FuncAssociate 2.0. [26].

Our GEA revealed that the four tissue types were each enriched for multiple gene ontology categories (Table 1 and Table S1). Because we analyzed both whole tissue and isolated adipocytes, we were also able to infer whether the differentially expressed genes arise from predominantly either the adipocytes themselves, or from the more heterogeneous adipose tissue mixtures.

In sqAds, our GEA revealed a striking enrichment of pattern specification genes (Table 1). Because the current GO classifications do not include all known homeobox genes, we manually referenced our differentially expressed genes against a comprehensive list of human homeobox genes [27]. Compared to pcAds, sqAds demonstrated increased expression of 12 homeobox genes, including PAX3, HOXA10, HOXA9, and HOXB7 (Table 1). All of the homeobox genes that were relatively higher in sqAds were also increased in SQAT vs. PCAT (Table S2). For example, PAX3 was 3.3-fold (p = 0.00002) increased in sqAds vs. pcAds and 3.8-fold (p = 0.0004) increased in SQAT vs. PCAT.

When we looked at homeobox expression in the pericardial depot, we found significantly increased expression of two homeobox genes, HOXA2 (2.53-fold, p = 0.00012) and SATB1 (1.94-fold, p = 0.015), in both pcAds and PCAT (Table 1 and Table S2). In aggregate, the observed transcriptional patterns of these 14 homeobox genes constitute depot-specific signatures.

We also found that our pcAds were significantly enriched for immune response genes (p-adj < 0.001, Table 1 and Table S3), with 26 immune response genes found among the top 2500 genes. A similar result was seen in PCAT (p-adj = 0.005, Table S1). These genes included 7 chemokines, such as chemokine (C-C motif) ligand 4 (CCL4) which was overexpressed 2.7-fold (p < 0.0001) in pcAds and 2.9-fold (p = 0.0009) in PCAT. To better visualize the molecular relationships between these inflammatory genes, we plotted the overexpressed genes to KEGG pathways (Fig. 2), revealing that the chemokines upregulated in pcAds belong to the tumor necrosis factor (TNF), CXC and CC chemokines, and IL-1 families. Since expression levels were not significantly lower in pcAds compared to PCAT, it is possible that pericardial adipocytes are a primary site for the synthesis of inflammatory mediators. We thus sought to determine, using qPCR, the expression of CCL4 in isolated pcAds and isolated pericardial stromal vascular fraction (SVF). We found 12.5-fold (p = 0.005) higher expression of CCL4 in the pericardial SVF vs. the pcAds fraction. Altogether, these data demonstrate that pcAds express inflammatory mediators but not to the same degree as the SVF, which contains immune and inflammatory cells. In contrast to PCAT, our GEA revealed that SQAT was significantly enriched in many metabolism-related GO categories (Table S1), including oxidoreductase activity (p-adj = 0.0002) and lipid metabolic processes (p-adj = 0.0008). KEGG pathway mapping did not identify any recognized, adipose-tissue specific pathways.

To validate the differential gene expression patterns identified by our microarray analysis, we performed qPCR analysis on patient-matched sqAds and pcAds samples (Fig. 3). For this analysis, we selected homeobox genes HOXA9 and HOXB7; and the adipocyte identity gene leptin. For all three genes, the expression trend matched our microarray results. Lastly, we confirmed that a core set of adipocyte identity and function genes was not differentially expressed in pcAds vs. sqAds (Table S4). The genes we selected included the well-studied adipocyte identity maintenance factors perlipin (PLIN1) and cell death-inducing DFFA-like effector c (CIDEc); the transcription factors Peroxisome proliferator-activated receptor gamma (PPARγ) and the CCAAT/enhancer binding proteins (CEBPA, CEBPB, CEBPD); the lipid catabolism enzymes lipoprotein lipase (LPL) and hormone-sensitive lipase (LIPE); the adipocyte anabolic enzymes fatty acid binding protein 4 (FABP4) and diacylglycerol O-acyltransferase 1 (DGAT1); and the adipokines leptin (LEP), adipin (CFD), and adiponectin (ADIPQ). We also considered the brown fat identity gene uncoupling protein 1 (UCP1) and found ±0.50 fold change between the sample classes.

**Discussion**

Adipose tissue is an active, multifunctional organ whose different depots in the human body most likely serve different functions. For example, a strong correlation exists between excess omental adipose tissue and the development of cardiovascular disease [7][8][9]. While early evidence suggested that pericardial adipose tissue functions in pericardial inflammation, these studies were purely radiographic in nature [16][17][18][19][20][21]. There is evidence, however, that PCAT can directly affect cardiac function as there is a several studies have found an association
between PCAT volume and atrial fibrillation [28][29]. PCAT may also serve an endocrine role, as increased PCAT volume is positively associated with liver fat and insulin sensitivity after diet-induced weight loss [30].

Our study is the first transcriptional microarray analysis of gene expression differences between PCAT and SQAT and, importantly, between pcAds and sqAds. We found that: 1) pericardial and subcutaneous fat depots, as well as isolated pericardial and subcutaneous adipocytes, express unique patterns of homeobox genes, and 2) pericardial adipose tissue and isolated pericardial adipocytes both overexpress chemokines that promote atherosclerosis. In contrast, subcutaneous adipose tissue is enriched for metabolism-related genes. The finding of a specific pericardial-specific homeobox signature fits with recent reports of depot-dependent homeobox signatures in humans. Gesta et al. compared human SQAT to visceral adipose tissue (VAT) and found that SQAT overexpressed SHOX2, HOXC9, EN1, and GPC4, while VAT overexpressed factors including HOXA5, TBX15, and HOXC8 [31]. Our data confirm that SHOX2 and HOXC9 are upregulated in SQAT (Table 2). Vohl et al. reported that HOXA10 and HOXC6 were upregulated in human SQAT vs. VAT; [32] this pattern is also confirmed by our data (Table 1).

The functional importance of these signatures needs further study. One hypothesis is that these homeobox signatures reflect distinct cell lineages [33][34]. If so, such lineage differences, if extensive, may imply that excess adiposity of different fat depots (e.g. excess omental fat vs. excess pericardial fat) would not respond equally well to single therapies. Rather than simply being

Figure 1. Experimental overview and clustering results. (A) Experimental design. (B) Clustering of isolated pericardial and subcutaneous adipocytes following removal of date-related batch effects by principal component analysis. (C) In our unbiased GEA, differentially expressed genes in each sample group (SQAT, sqAds, pcAds, and PCAT) were identified by preprocessing the data, performing principal component analysis, and setting thresholds of p-value<0.05 and FDR <0.25. Unique genes corresponding to candidate probesets were analyzed with FuncAssociate 2.0 (see Methods).

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Table 1. Enriched Gene Ontology (GO) Categories in sqAds and pcAds identified by GEA.

| # of Genes | P-adjusted | GO ID     | GO Category                                      |
|------------|------------|-----------|--------------------------------------------------|
|            |            | GO:0009952| anterior/posterior pattern formation             |
|            | 6          | 0.004     |                                                   |
|            | 6          | 0.026     | regionalization                                   |
|            | 6          | 0.03      | protein tetramerization                           |
|            | 8          | 0.047     | protein homooligomerization                       |
|            | 10         | 0.006     | sequence-specific DNA binding                     |
|            | 12         | 0.003     | transcription factor activity                     |
|            | 19         | 0.02      | anatomical structure morphogenesis                |
|            | 48         | 0.002     | developmental process                             |

**Upregulated in sqAds**

| # of Genes | P-adjusted | GO ID     | GO Category                                      |
|------------|------------|-----------|--------------------------------------------------|
| 6          | 0.004      | GO:0003002| regionalization                                   |
| 6          | 0.026      | GO:0051262| protein tetramerization                           |
| 6          | 0.03       | GO:0051260| protein homooligomerization                       |
| 8          | 0.047      | GO:0043565| sequence-specific DNA binding                     |
| 10         | 0.006      | GO:0003700| transcription factor activity                     |
| 12         | 0.003      | GO:009653 | anatomical structure morphogenesis                |
| 19         | 0.02       | GO:0032502| developmental process                             |
| 48         | 0.002      |           |                                                   |

**Upregulated in pcAds**

| # of Genes | P-adjusted | GO ID     | GO Category                                      |
|------------|------------|-----------|--------------------------------------------------|
| 27         | 0          | GO:0048583| regulation of response to stimulus               |
| 38         | 0          | GO:0042127| regulation of cell proliferation                 |
| 39         | 0          | GO:0002376| immune system process                            |
| 62         | 0          | GO:0048522| positive regulation of cellular process           |
| 67         | 0          | GO:0048518| positive regulation of biological process         |
| 15         | 0.001      | GO:0050865| regulation of cell activation                    |
| 24         | 0.001      | GO:0008284| positive regulation of cell proliferation         |
| 26         | 0.001      | GO:0006955| immune response                                  |
| 14         | 0.005      | GO:0006935| chemotaxis                                       |
| 14         | 0.005      | GO:0042330| taxis                                            |
| 24         | 0.01       | GO:0009605| response to external stimulus                    |
| 27         | 0.013      | GO:0005576| extracellular region                             |
| 32         | 0.014      | GO:0035466| regulation of signaling pathway                  |
| 5          | 0.02       | GO:0042692| muscle cell differentiation                      |
| 11         | 0.023      | GO:0009897| external side of plasma membrane                 |
| 26         | 0.026      | GO:0051239| regulation of multicellular organismal process   |
| 44         | 0.038      | GO:0042221| response to chemical stimulus                    |
| 11         | 0.04       | GO:0046649| lymphocyte activation                            |
| 13         | 0.042      | GO:0001775| cell activation                                  |
| 21         | 0.044      | GO:0002682| regulation of immune system process              |

Figure 2. Chemokines overexpressed in pcAds. Red boxes represent overexpressed molecules. Double-gray lines indicate cell surface membrane; boxed proteins overlying double-gray lines are receptors.

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markers, homeobox genes could also function in adipose tissue function. Dankel et al. reported that profound fat loss in humans was correlated with strong upregulation in SQAT of HOXA9, HOXC6, and several other homeobox genes [35]. These genes could represent novel targets for modifying adipogenesis and, by extension, obesity.

The heterogeneous nature of different adipose depots is highlighted by the functional differences in this study. Our data show that both pericardial adipose tissue and isolated pericardial adipocytes are immunologically active, overexpressing several chemokines (Fig. 2), many of which have been implicated in the development of atherosclerosis and coronary artery disease (CAD). The CC subfamily chemokines overexpressed in our pcAds (CCL4, and CCL5) are found at high levels in atherosclerotic lesions [36][37][38][39][40]. CCL5 has been indirectly shown to be atherogenic: antagonism of its receptor with a methionine-retaining CCL5 isoform slows atherosclerosis in vivo [41]. Although it was not included in Fig. 2, CCL2 also trended towards overexpression in pcAds (1.5-fold overexpression, p-value = 0.017) but had an FDR of 0.26. When CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), was deleted in transgenic mice, macrophages were not recruited into atherosclerotic plaques and the atherosclerotic lesions were 60–70% smaller [42]. A similar phenotype was observed in mice deficient for CCR2, the receptor for CCL2 [43].

The overexpressed CXC subfamily chemokines also have direct, pro-atherosclerotic effects in vivo [44]. CXCL1 was implicated in the transition of early fatty streaks to intermediate atherosclerotic lesions [45]. In porcine models it induced endothelial dysfunction by downregulating nitric oxide synthase protein levels [46]. CXCL5 appears to elevate serum levels of CXCL1 and may be an indirect factor in atherogenesis [47].

In the interleukin family, IL-1β (1.7-fold overexpressed in pcAds) is known to increase LDL binding to atherosclerotic lesions which then induces more IL-1 production [48][49]. Conversely, atherosclerosis-prone mice that were deficient in IL-1β had significantly smaller atherosclerotic plaques [50]. Given this mechanistic data, the overexpression of these chemokines in

![Figure 3. Quantitative PCR confirms differential expression of homeobox genes HOXA9 and HOXB7; and adipocyte-identity gene leptin (LEP) in patient-matched, isolated subcutaneous and pericardial adipocytes.](image-url)
pcAds strongly suggests a direct role for pericardial adipocytes in the development of coronary artery atherosclerosis.

Our study highlights a number of new areas for further research. It is unclear if these depot-specific homeobox gene signatures are residual from differentiation, or continue to drive expression of genes important for defining tissue identity. Compelling follow-up studies would include identification of downstream targets and pathways of these homeobox genes. More research is also needed to confirm local and perhaps systemic levels of pericardial adipocyte-secreted chemokines, and further on, to identify the pathways that they activate.

In conclusion, pericardial and subcutaneous fat depots express unique homeobox signatures and serve different functional roles. As the first microarray study of the pericardial adipose depot, we show that pericardial adipocytes preferentially express pro-atherosclerotic factors and may therefore function in the development of cardiovascular disease. Future research should aim to further characterize these emerging, depot-specific functions.

Materials and Methods

This Project has been reviewed and approved by the Brigham & Women’s Hospital Institutional Review Board, Assurance #FWA00000484. During the review of this Project, the IRB specifically considered (i) the risks and anticipated benefits, if any, to subjects; (ii) the selection of subjects; (iii) the procedures for securing and documenting informed consent; (iv) the safety of subjects; and (v) the privacy of subjects and confidentiality of the data. Written consent was obtained from all participants.

Between June 2009 and April 2010, 21 patients who underwent elective coronary artery bypass grafting and/or cardiac valve replacement surgeries participated in the study. With the exception of prisoners and subjects with surrogate decision makers, there were no exclusion criteria or patient classes. No enrolled patients were excluded. Approximately 3 mL of pericardial adipose tissue (PCAT) and 3 mL of subcutaneous adipose tissue (SQAT) from the anterior chest wall were harvested from patients undergoing elective coronary artery bypass grafting and/or cardiac valve replacement surgery. Epicardial adipose tissue was not harvested. As adipose degrades quickly after surgical removal, all samples were retrieved immediately from the operating room and not harvested. As adipose degrades quickly after surgical removal, all samples were retrieved immediately from the operating room after being harvested. The samples were sectioned and assigned to either whole tissue analysis or isolated adipocyte analysis. For PCAT and SQAT samples, RNA was immediately extracted via submersion in 1 ml of Trizol, mechanical lysis using an RNase free pestle (Kimble Chase Kontes), addition of 200 µl chloroform, and centrifugation (10,000 g, 15 minutes, 4°C). The supernatant was extracted and purified with RNeasy Mini Columns (Qiagen) to minimize the potential impact of any genomic DNA. RNA was then extracted from the sqAds and pcAds samples in the same fashion as SQAT/PCAT samples. To quantify the purity of the isolated adipocytes, 100 µL of freshly isolated adipocytes was mixed with 150 µL of PBS containing Hoechst 33258 nuclear stain (Invitrogen, 10 mg/mL, diluted 1:5000) and Bodipy stain (Invitrogen, 5 mM diluted 1:20,000). 50 µL of this mix was mounted directly onto slides (Fischer) using Aqua Poly/Mount (Polysciences). Cells and nuclei were counted using a Nikon Eclipse Ti microscope, and representative images were captured using NIS-Elements software package (Nikon, version 3.10).

All RNA samples were analyzed for quality using an Agilent 2100 Bioanalyzer. Suitable samples were hybridized to Affymetrix Human Genome U133 Plus 2.0 Arrays. The arrays were processed by the Stowers Institute for Medical Research Bioinformatics Core Facility. All data is MIAME compliant and the raw data has been deposited in the Gene Expression Omnibus database (accession number GSE26339).

Raw expression values were analyzed with GenePattern 2.0 [51]. Data were normalized using Robust Multiarray Averaging (RMA) with median scaling, quartile normalization, and背景 correction. The resulting datasets were preprocessed to remove probesets whose minimum fold change (maximum gene expression value divided by the minimum value) was <2, or whose difference between maximum and minimum values was less than 100. The preprocessed data was clustered in an unbiased hierarchical fashion, with Pearson correlation and pairwise complete-linkage.

RNA samples were collected, extracted, amplified and hybridized in several separate batches over the period of 10 months. To explore the influence of batch effects, principal component analysis was performed using the prcomp function in R (2.9.1). The first principal component, explaining 38% of the variance, corresponded to the date of sample processing, with samples processed in June 2009 grouped separately from all other samples. The contribution of this component was removed from normalized intensities prior to further analysis.

Unbiased GEA was performed using FuncAssociate 2.0 (http://llama.mshri.on.ca/funcassociate/), which uses a Fisher’s exact test to assess enrichment and a resampling approach to correct for multiple hypotheses. For each of the sample populations, a false discovery rate (FDR) of 0.25 was set as the threshold. We also performed sensitivity analysis to ensure that FDR thresholds between 0.2 and 0.3 did not change the identify of enriched gene sets. The differentially expressed probesets were uploaded into FuncAssociate 2.0 as ordered lists. Analysis was performed using the hgc_symbol namespace, with 1000 permutations for p-value estimation and a p-value cutoff of 0.05.

Pathways involving upregulated genes in significantly enriched GO categories were identified using DAVID and KEGG [52][53][54].

For qPCR, expression levels of HOXA9 and HOXB7 were normalized to the housekeeping gene beta-2-microglobulin (B2M) and measured via Taqman Assay (Applied Biosciences). Both the pcAds and sqAds samples were patient-matched (Patient 15). Leptin expression levels in matched samples (from Patient 5) were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and measured via Quantitect SYBR Green PCR Kit (Qiagen). To minimize the potential impact of any genomic DNA contamination, all primers were designed and verified to span multiple exons. Three technical replicates were performed for each sample. Error bars were computed by adding and subtracting 1 unit standard deviation of the delta Ct values from calibrated delta Ct values. P-values were calculated in Microsoft Excel 2007 using 2-tailed heteroscedastic Student’s t-tests.

Supporting Information

Figure S1 Purity of isolated adipocytes. (A) Results of nuclear and cell counts in isolated adipocyte fraction; the fraction is 96.7%
pure. (B) Representative image of isolated sqAds demonstrating 1:1 association of nuclei with adipocytes. (TIF)

**Figure S2** Unbiased hierarchical clustering of isolated pericardial adipocytes (pcAds) and isolated subcutaneous adipocytes (sqAds). Sections of the dendrogram in red indicate samples clustering of pcAds and sqAds samples processed in June 2009. (TIF)

**Table S1** Enriched Gene Ontology categories in SQAT and PCAT. (DOCX)

**Table S2** Upregulated Homebox Genes in SQAT and PCAT. (DOCX)

**Table S3** Upregulated Immune Response genes in pericardial adipocytes. (DOCX)

**Table S4** Expression of core adipocyte function genes in isolated pericardial adipocytes (pcAds) vs. isolated subcutaneous adipocytes (sqAds). (DOCX)

**Author Contributions**
Conceived and designed the experiments: JC LP JDW BRR CAC. Performed the experiments: FHL GM JG TA AK LP JDW BRR CAC. Analyzed the data: FHL, RCD GW TA AK CAC. Contributed reagents/materials/analysis tools: FHL RCD JG BRR CAC. Wrote the paper: FHL, RCD GM AK CAC.

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