Network Analysis of Human In-Stent Restenosis

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**Background**—Recent successes in the treatment of in-stent restenosis (ISR) by drug-eluting stents belie the challenges still faced in certain lesions and patient groups. We analyzed human coronary atheroma in de novo and restenotic disease to identify targets of therapy that might avoid these limitations.

**Methods and Results**—We recruited 89 patients who underwent coronary atherectomy for de novo atherosclerosis (n = 55) or in-stent restenosis (ISR) of a bare metal stent (n = 34). Samples were fixed for histology, and gene expression was assessed with a dual-dye 22 000 oligonucleotide microarray. Histological analysis revealed significantly greater cellularity and significantly fewer inflammatory infiltrates and lipid pools in the ISR group. Gene ontology analysis demonstrated the prominence of cell proliferation programs in ISR and inflammation/immune programs in de novo restenosis. Network analysis, which combines semantic mining of the published literature with the expression signature of ISR, revealed gene expression modules suggested as candidates for selective inhibition of restenotic disease. Two modules are presented in more detail, the procollagen type 1 α2 gene and the ADAM17/tumor necrosis factor-α converting enzyme gene. We tested our contention that this method is capable of identifying successful targets of therapy by comparing mean significance scores for networks generated from subsets of the published literature containing the terms “sirolimus” or “paclitaxel.” In addition, we generated 2 large networks with sirolimus and paclitaxel at their centers. Both analyses revealed higher mean values for sirolimus, suggesting that this agent has a broader suppressive action against ISR than paclitaxel.

**Conclusions**—Comprehensive histological and gene network analysis of human ISR reveals potential targets for directed abrogation of restenotic disease and recapitulates the results of clinical trials of existing agents. (Circulation. 2006;114:2644-2654.)

**Key Words:** atherosclerosis ■ paclitaxel ■ restenosis ■ sirolimus

Since Sigwart et al first reported the use of a metallic stent as an adjunct to balloon angioplasty for the treatment of flow limitation in coronary arteries, the issue of in-stent restenosis (ISR) has been the focus of attention of interventional cardiologists and vascular biologists alike. Incidence in published studies ranges between 10% and 60%, and despite early attempts with systemic therapy and direct stenting, only the more recent use of stents coated with antiproliferative agents has made a significant impact on the disease. Indeed, such is the benefit of these drug-eluting stents that there has been almost universal adoption, at least in the United States, for eligible lesions.

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However, although sirolimus- or paclitaxel-eluting stents have consistently demonstrated lower rates of ISR compared with the bare metal control in a broad spectrum of lesion types, sufficient data for specific lesion subsets such as saphenous vein grafts, bifurcation lesions, and acute myocardial infarctions are still missing. In addition, concerns about the incidence of late stent thrombosis, related to delayed stent endothelialization, have tempered enthusiasm. Finally, although the occurrence of ISR within drug-eluting stents is greatly reduced, it remains between 3% and >25% in published trials.
For these reasons and others, there is continued interest in new pharmacotherapeutic agents, suitable for stent-based delivery to the coronary vasculature, that better address the needs of this particular vascular bed. One approach lies in a more sophisticated understanding of restenosis biology. Although classic studies established the role of platelet-facilitated inflammation, migration, dedifferentiation, and proliferation of smooth muscle cells and the elaboration of extracellular matrix (neointimal hyperplasia), more in-depth characterization has been limited by access to human coronary atheromatous material. Meanwhile, more recent high-dimensional genomic and proteomic tools allow not only a more comprehensive view of the disease at the transcriptional level but also a perspective not limited by preconceived speculation as to the process of interest. Moreover, although light microscopy offers a morphological examination of the cell types involved at the tissue level, molecular profiling can offer insight into the ongoing biological program of those cells.

For these reasons and others, there is continued interest in new pharmacotherapeutic agents, suitable for stent-based delivery to the coronary vasculature, that better address the needs of this particular vascular bed. One approach lies in a more sophisticated understanding of restenosis biology. Although classic studies established the role of platelet-facilitated inflammation, migration, dedifferentiation, and proliferation of smooth muscle cells and the elaboration of extracellular matrix (neointimal hyperplasia), more in-depth characterization has been limited by access to human coronary atheromatous material. Meanwhile, more recent high-dimensional genomic and proteomic tools allow not only a more comprehensive view of the disease at the transcriptional level but also a perspective not limited by preconceived speculation as to the process of interest. Moreover, although light microscopy offers a morphological examination of the cell types involved at the tissue level, molecular profiling can offer insight into the ongoing biological program of those cells.

In the present article, we assess the form and function of coronary vascular disease through light microscopy and nearly genome-wide gene expression to offer novel insights into the process of ISR. Furthermore, we use an original integrative analysis to identify key components of biological pathways that may be candidates for future therapeutics.

Finally, we test our approach against results of recently published clinical trials comparing currently available elution agents.

**Methods**

The experimental approach is summarized in Figure 1.

**Patient Recruitment and Tissue Harvest**

Eighty-nine patients with significant coronary lesions suitable for atherectomy were recruited at the HELIOS Heart Center Siegburg (Siegburg, Germany). Standard premedication with oral aspirin, clopidogrel, and intracoronary heparin was given. Femoral arterial access was gained with 6F or 8F introducers and a sterile, over-the-wire technique. Diagnostic angiography was then performed with standard catheters, and the coronary anatomy was defined. If a significant, flow-limiting stenosis was identified in a moderate-sized vessel, an extra support guidewire was deployed, and longitudinal atherectomy was performed with the Silverhawk atherectomy catheter (Foxhollow Technologies, Redwood City, Calif). All procedures were concluded with standard percutaneous coronary transluminal angiography/stenting at the operator’s discretion. Extracted specimens were divided and stored immediately in both liquid nitrogen and formalin. Of 89 samples, 36 had RNA of sufficient quality for hybridization, and 47 arrays were judged to be of sufficient quality for analysis after rigorous quality control.

The study was approved by the Stanford University Institutional Review Board. All patients gave written, informed consent.
Development of Oligonucleotide Microarray Platform

A custom microarray was developed by our laboratory in collaboration with Agilent Technologies (Palo Alto, Calif). Its development has been described in detail previously.9 In brief, a combination of techniques based on experimental cell culture and data mining was used to provide a comprehensive catalog of vascular and atherosclerosis-related genes. Probes derived from this list were combined with those from Agilent human catalog arrays 1A and 1B to create the final 22 000–feature microarray.

RNA Isolation and Quantification

RNA was isolated using standard techniques based on guanidium thiocyanate as described previously.9 Extensive quality control testing of all RNA was carried out. Samples were quantified with the NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE), and RNA integrity was assessed with the 2100 Bioanalyzer System and RNA 6000 Pico LabChip Kit (Agilent Technologies).

Direct Labeling and Oligonucleotide Array Hybridization

Our protocol has been described in detail previously.9 In brief, 2 to 5 μg RNA for sample and reference was used. A mixture of 80% HeLa RNA and 20% human umbilical vein endothelial cell RNA was used for reference because it had previously been shown to maximize feature fluorescence for this array. After linear amplification, fluorescent-labeled cDNA was reverse transcribed from the RNA with SuperScript II (Invitrogen, Carlsbad, Calif) and either cyanine 3-dCTP or cyanine 5-dCTP (Perkin-Elmer/NEN, Boston, Mass). After purification, hybridization continued for 16 hours at 60°C. Arrays were scanned with the G2565AA Microarray Scanner System, and features were extracted as previously described. Excluded features were estimated with the K nearest neighbors imputation method of Troyanskaya et al.10

Histology

Tissue samples were fixed in 10% neutral buffered formalin, processed routinely through graded alcohols and clearing reagent, and embedded in paraffin blocks. Tissue sections (5 μm thick) were

### TABLE 1. Patient Characteristics for the 47 Samples Included in the Genomic Analysis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>De Novo Restenosis (n=32)</th>
<th>ISR (n=15)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66±11.85</td>
<td>70±7.66</td>
<td>...</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>21 (84)</td>
<td>9 (64.28)</td>
<td>0.75</td>
</tr>
<tr>
<td>Unknown</td>
<td>...</td>
<td>1 (7.14)</td>
<td>...</td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>21 (84)</td>
<td>14 (100)</td>
<td>0.07</td>
</tr>
<tr>
<td>Asian</td>
<td>1 (4)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (12)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 (52)</td>
<td>10 (71.42)</td>
<td>0.12</td>
</tr>
<tr>
<td>No</td>
<td>7 (28)</td>
<td>2 (14.28)</td>
<td>...</td>
</tr>
<tr>
<td>Unknown</td>
<td>5 (20)</td>
<td>2 (14.28)</td>
<td>...</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>15 (60)</td>
<td>12 (85.71)</td>
<td>0.03</td>
</tr>
<tr>
<td>No</td>
<td>6 (24)</td>
<td>2 (14.28)</td>
<td>...</td>
</tr>
<tr>
<td>Unknown</td>
<td>4 (16)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1 (4)</td>
<td>2 (14.28)</td>
<td>0.23</td>
</tr>
<tr>
<td>No</td>
<td>11 (44)</td>
<td>8 (57.14)</td>
<td>...</td>
</tr>
<tr>
<td>Unknown</td>
<td>13 (52)</td>
<td>4 (28.57)</td>
<td>...</td>
</tr>
<tr>
<td>Tobacco, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5 (20)</td>
<td>4 (28.57)</td>
<td>0.44</td>
</tr>
<tr>
<td>No</td>
<td>20 (80)</td>
<td>10 (71.42)</td>
<td>...</td>
</tr>
<tr>
<td>Previous acute coronary syndrome, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (44)</td>
<td>12 (85.71)</td>
<td>0.005</td>
</tr>
<tr>
<td>No</td>
<td>4 (16)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Unknown</td>
<td>10 (40)</td>
<td>2 (14.28)</td>
<td>...</td>
</tr>
<tr>
<td>Drug therapy, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>10 (40)</td>
<td>7 (50)</td>
<td>0.34</td>
</tr>
<tr>
<td>Angiotensin receptor blockers</td>
<td>0</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>β-Blockers</td>
<td>15 (60)</td>
<td>9 (64.28)</td>
<td>0.53</td>
</tr>
<tr>
<td>Ca channel antagonist</td>
<td>3 (12)</td>
<td>2 (14.28)</td>
<td>0.65</td>
</tr>
<tr>
<td>Statin</td>
<td>14 (56)</td>
<td>11 (78.57)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Age is given as mean±SD. ACE indicates angiotensin-converting enzyme.
cut and stained with hematoxylin and eosin, Gomori’s trichrome, and elastic van Gieson stain. Sections were classified as de novo atherosclerosis or ISR and evaluated microscopically. Samples were graded according to the presence of certain key features: low cellularity (abundant dense staining of collagen with sparse cells), high cellularity (rich stellate or spindle cells within a loose extracellular matrix or limited amount of collagen), lipid deposit (focal area with clusters of foam cells, cholesterol clefts, pale granular materials, and acellular debris), and inflammatory focus (the presence of clusters or aggregates of mononuclear cells, including monocytes, macrophages, and lymphocytes).

Analytical Methods

Proportions of categorical demographic and histological variables were compared through the use of Fisher’s exact test.

Our primary difference measure for genomic analysis was the significance analysis of microarrays. With this measure, a significance score is calculated as follows:

$$y_{ik} - y_{jk}/s_i + s_0,$$

where \(y_{ik}\) is the mean expression of gene \(i\) in the ISR group, \(y_{jk}\) is the mean expression of gene \(i\) in the de novo atherosclerosis group, \(s_i\) is the standard deviation of repeated expression measurements, and \(s_0\) is a small positive constant. The metric is a modified \(t\) statistic (labeled \(d\) because of the addition of a small positive constant to the denominator). Multiple testing is accounted for by bootstrap permutation. Results from difference analysis are displayed using heatmap software developed by the authors and freely available to the academic community (http://quertermous.stanford.edu/heatmap.htm).

Differentially expressed genes were analyzed in the context of biological networks and to make a distinction from the hub gene concept, in which connections are derived from a form of network analysis centered around correlation of gene expression. In our method, we use semantic mining of the published literature. An association network is derived from text mining of Medline abstracts and association identified between any 2 genes if they appear in the same sentence as an interaction verb as defined by our user context file. A series of subnetworks (independent of our experimental data) is then generated, and the expression values and significance of genes in our analysis overlaid visually and mathematically on these networks. An overall significance score for each subnetwork is calculated by using the mean significance (\(d\)) score value, or the cumulative sum of significance (\(d\)) score values, for all members. Higher scores thus indicate greater significance of the subnetwork.

To test our hypothesis that connectivity analysis is capable of identifying targets that may lead to more efficacious therapies, we took advantage of the recent publication of multiple head-to-head trials of paclitaxel- and sirolimus-eluting stents. These clinical trials demonstrated a small but consistent and significant benefit of sirolimus over paclitaxel in the inhibition of ISR. We posited that our methods should be capable of predicting this result. Thus, 2 abstract subdatabases were generated by entering the following queries into Medline: “(rapamycin OR sirolimus) NOT paclitaxel”.

We called these genes nexus genes to emphasize their central role in biological networks and to make a distinction from the hub gene concept, in which connections are derived from a form of network analysis centered around correlation of gene expression. In our method, we use semantic mining of the published literature. An association network is derived from text mining of Medline abstracts and association identified between any 2 genes if they appear in the same sentence as an interaction verb as defined by our user context file. A series of subnetworks (independent of our experimental data) is then generated, and the expression values and significance of genes in our analysis overlaid visually and mathematically on these networks. An overall significance score for each subnetwork is calculated by using the mean significance (\(d\)) score value, or the cumulative sum of significance (\(d\)) score values, for all members. Higher scores thus indicate greater significance of the subnetwork.

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<table>
<thead>
<tr>
<th>Lesions</th>
<th>De Novo Cases, n (%)</th>
<th>ISR Cases, n (%)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low cellularity</td>
<td>45 (81.8)</td>
<td>9 (26.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>High cellularity</td>
<td>10 (18.2)</td>
<td>25 (73.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lipid deposit</td>
<td>14 (25.5)</td>
<td>1 (2.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Inflammatory focus</td>
<td>21 (38.2)</td>
<td>3 (8.8)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Thrombus</td>
<td>11 (20)</td>
<td>4 (11.8)</td>
<td>0.25</td>
</tr>
<tr>
<td>Calcification</td>
<td>3 (5.5)</td>
<td>1 (2.9)</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Cellularity was significantly higher in the ISR group; lipid deposits and inflammatory foci were more common in the de novo group. There was no difference in the frequency of thrombus or calcification.

TABLE 2. Histological Features of 55 De Novo Restenotic and 34 ISR Lesions
and "paclitaxel NOT sirolimus NOT rapamycin." Network analysis was then carried out within these abstract databases, and the overall significance of each subnetwork was calculated as detailed above. The means for sirolimus and paclitaxel groups were compared by Student's t test. In a separate analysis, we incorporated the terms "sirolimus" and "paclitaxel" directly as interacting gene terms to generate 2 large additional networks. Overall significance also was compared for these networks.

Pathway interactions are mapped with Cytoscape (version 2.2)\(^4\) with the Agilent literature search plug-in (version 2). Using a separate routine, we overlaid significance levels as colors and depicted raw data as a short "heat strip" below each node.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Patient Population**

Patient characteristics for the 47 samples in the genomic analysis are detailed in Table 1. Although overall similar, some demographic variables were significantly different between the 2 populations. Hypercholesterolemia was more common in the ISR group (15 of 32 versus 12 of 15; \(P=0.03\)). This was reflected in greater use of statins (14 of 32 versus 11 of 14; \(P=0.07\)). In addition, a history of acute coronary syndrome was much more common in the ISR group (11 of 32 versus 12 of 15; \(P=0.005\)). The prevalence of known diabetes was low in our cohort and not different between groups (1 of 32 versus 2 of 15; \(P=0.23\)), but this may reflect the high level of unknowns in this category.

**Gross Specimen and Histological Analysis**

Lesions were classified as de novo restenosis or ISR by the operator at the time of catheterization. Samples were weighed before tissue processing (mean, 1.22±1.02 mg; range, 0.1 to 4.77 mg; Figure 2A and 2B). Histological analysis (Figure 2C through 2H and Table 2) revealed significantly greater frequency of low-cellularity lesions in the de novo group (81.8% in de novo versus 26.5% in ISR; \(P<0.0001\)). There was a corresponding higher frequency of high-cellularity lesions within the intima of ISR lesions (73.5% in ISR versus 18.2% in de novo; \(P<0.0001\)). De novo lesions exhibited classically recognized features of atherosclerosis to a greater extent than ISR lesions: lipid deposit, 25.5% in de novo versus 2.9% in ISR (\(P=0.001\)); and inflammatory infiltrates, 38.8% in de novo versus 8.8% in ISR (\(P=0.0004\)). There was no significant difference in the incidence of calcification or thrombosis between groups.

**Gene Expression Analysis**

Comparison using the significance analysis of microarrays revealed highly significant differences between de novo lesions and ISR lesions. Plotting the false discovery characteristics (Figure 3A and 3B) reveals a nadir rate of false discovery at \(\approx200\) genes (0.77 genes per 207 genes depicted, \(<1\) falsely significant gene, 0.4% false discovery rate). In addition, at this level of false discovery, all significant genes had greater expression in the ISR group (Figure 3B and 3C).

**Ontology Analysis**

Although gene lists are informative, systems analysis lends structure to large data sets. Patterns of gene expression within the most upregulated genes in each group (t test) were assessed for overrepresentation in the context of molecular function, biological process, and cellular component (Gene Ontology Consortium). Significant gene ontology terms are depicted in Figure 4 (all \(P<1.5\times10^{-5}\)). As shown, the primary molecular signature differentiating de novo atherosclerosis from ISR is an immune/inflammatory one (Figure 4A and 4B). All significant terms in genes upregulated in de novo atherosclerosis are children of the "response to stress" and the "response to external stimulus" parents. In contrast, the primary molecular signature of ISR is cell growth and extracellular matrix (Figure 4A and 4B). We found no evidence of overrepresentation of inflammatory ontologies compared with de novo atherosclerosis in our data.
Figure 3. Continued
Network Analysis

We generated subnetworks using text mining of Medline abstracts and awarded each an overall significance score based on the average significance of network members within our experimental data set (Table 3). Subnetworks were explored using Cytoscape, and sentences were curated for the most promising candidate networks. Example subnetworks are reported in Table 3 and illustrated in Figure 5. The analysis provided a highly enriched source of candidates, both expected (collagen genes, fibroblast receptor gene, interleukin-8) and unexpected (ADAM genes, nm23 protein gene). Figure 5 shows the ADAM17 subnetwork. ADAM17 is the tumor necrosis factor (TNF)-α converting enzyme, which regulates inflammation. Other notable members of the ADAM17 subnetwork include other disintegrin and metalloproteinase domain (ADAM) proteins, inducible nitric oxide synthase, tissue inhibitors of matrix metalloproteinases, and endothelial growth factor.

Targets for inhibiting ISR include differentially regulated networks with nexus genes highly upregulated in this tissue. Several are reported in Table 3, and one is shown in Figure 5B. The procollagen type I α2 gene (COL1A2) was as highly upregulated as any nexus gene in the top 50 subnetworks (d
The subnetwork contained other collagen genes (COL1A1) and regulatory elements (Sp1), as well as key mediators of inflammation: interferon-γ, transforming growth factor-β, major histocompatibility class II transactivator, interleukin-4, fundamental signaling molecules (PI3 kinase, mitogen-activated protein kinase 8), and matrix metalloproteinase 13.

To test the idea that useful targets of therapy could be identified by our approach, we took advantage of recently published head-to-head clinical trials of 2 agents effective against ISR. Networks were generated using subsets of the published literature that contained the terms “sirolimus or rapamycin NOT paclitaxel” or the reverse as noted above. The paclitaxel search generated 11,136 abstracts; the sirolimus network contained more gene associations. The sirolimus search generated 4,854 abstracts. Despite fewer abstracts, the overall significance levels of subnetworks with gene associations. The overall significance level among its subnetworks. As shown, the mean significance level of subnetworks in the sirolimus agents was more effective should demonstrate a higher mean significance level of subnetworks in the sirolimus group was significantly higher than for the paclitaxel group (P=0.028), suggesting that abstracts that included sirolimus in their content were more significantly different between de novo and ISR than those that included paclitaxel. Furthermore, when individual networks were generated by incorporating the terms sirolimus and paclitaxel directly as interacting nodes, the sirolimus network (518 associations) had an overall significance level (0.85) that was higher than that of paclitaxel (294 associations; overall significance level, 0.81). Together, these results support the idea that sirolimus has a broader efficacy of action in targeting genetic modules significantly different between de novo and ISR groups.

**Discussion**

We present here a comprehensive integrative analysis of ISR, applying systems tools to make specific predictions regarding new therapeutic targets. We also test our method against recently published clinical trials and find that our analysis supports the previously demonstrated broader efficacy of sirolimus over paclitaxel in the abrogation of ISR. Surprisingly few studies have examined the basic cellular processes of ISR, presumably because tissue is hard to acquire. Many studies have examined circulating markers or predictors, and a small number of studies have begun to describe allelic variation associated with the restenotic response. Indeed, a large National Institutes of Health study is currently underway to comprehensively assess the genomics of ISR. Identification of genes through the work described here significantly increases the number of candidate genes that can be investigated through these case-control genetic epidemiology studies.

In our histological analysis, we confirmed previous findings from cellular microscopy: ISR consists primarily of smooth muscle cells, seen in much greater number than in de novo atherosclerosis, and proteoglycan-rich matrix. Occasional focal collections of inflammatory cells and lipid pools are seen, but they are more common in de novo disease;
thrombus and calcification occur at the same frequency in each group. This description of cellular anatomy complements well the insight offered into cellular function by expression profiling.

Our technique of using gene expression profiles and network analysis is designed to identify genetic modules of interest. One of the most highly differentially regulated subnetworks in our analysis contained as its nexus ADAM17, the TNF-α converting enzyme. This molecule is believed to regulate inflammation via cleavage and release of transmembrane proteins from the cell surface, including L-selectin, TNF-α, and its receptors TNFR1 and TNFR2. As such, potentially, both proinflammatory and antiinflammatory effects could result. Release of soluble TNF would enhance inflammation, whereas release of a TNF receptor ectodomain might limit the effects of soluble TNF.23 In our analysis, TNF-α converting enzyme itself was highly downregulated (d score, −2.46), as were many members of the subnetwork, suggesting a role for this module in normal arterial function or in de novo atherosclerosis. Although little is known of the role of ADAM17/TNF-α converting enzyme in atherosclerosis, Canault et al24 recently reported the expression of active enzyme at the surface of macrophages isolated from atherosclerotic lesions of apolipoprotein E−/− mice fed a high-fat diet. Taken together, these findings argue strongly for future investigation of the TNF system dysregulation in general and ADAM17 in particular as a target for the interruption of atherosclerotic or restenotic processes.

Highly upregulated nexus genes in significantly differentially regulated networks are attractive targets for the interruption of ISR. COL1A2 (illustrated in Figure 5) is one such example and one that suggests potential targets for its own

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**Figure 6.** Subnets generated using text mining of Medline abstracts were awarded an overall significance score based on the average significance of network members in our experimental data set. Nodes are individual genes; edges are their connecting lines. Each edge represents a relationship revealed by semantic mining. Thicker edges imply a stronger relationship (more sentences with associations). A, The ADAM17 (TNF-α converting enzyme) subnetwork. B, The procollagen COL1A2 gene network. Below each node in the network is a heat strip representation of the raw gene expression data. Brown represents de novo restenosis; blue, ISR. The horizontal center equates to the median expression value for that gene so that the height of vertical bars above or below the line varies according to actual expression values. Nodes are colored according to their significance (bright red indicates highly significantly upregulated; bright green, highly significantly downregulated). C, Mean significance±SE for subnetworks generated from subsets of the published literature containing the terms “sirolimus” or “paclitaxel.”
suppression. For example, decoy Sp1-binding oligonucleotides have been shown to inhibit COL1A2 promoter activity both in cultured fibroblasts and in vivo in the skin of transgenic mice.\textsuperscript{25} Alternatively, interferon-\(\gamma\) represses collagen in a manner requiring a class II transactivator molecule.\textsuperscript{26} Both of these molecules are significantly downregulated in the COL1A2 subnetwork. Thus, a specific hypothesis generated by our data is that targeting of COL1A2, or one of the regulators shown, would abrogate ISR. Furthermore, we contend that this directed approach is more elegant than the global inhibition of cell proliferation induced by sirolimus, paclitaxel, or brachytherapy and thus is likely to result in fewer unwanted effects such as slow endothelialization and late stent thrombosis.

We have previously shown that high-dimensional techniques can identify targets for treatment in heart failure.\textsuperscript{27,28} To test the idea that such targets could be identified in vascular disease by our approach, we took advantage of recently published head-to-head clinical trials of 2 agents effective against ISR.\textsuperscript{4,13} Although differences in late lumen loss or target lesion revascularization in these trials could be explained by many factors, including those relating to deployment, stent design, polymer design, agent, or time course of elution, we hypothesized that an important factor was the agent itself. We found that in networks generated from subsets of the published literature focused on sirolimus or paclitaxel, the mean significance level of subnetworks in the sirolimus group was greater than that for the paclitaxel group. Thus, our method supports the results of multiple large-scale clinical trials.

Several limitations of our approach should be noted. First, tissue derived at 1 time point late in the disease process does not allow assessment of serial changes or the identification of tissue derived at 1 time point late in the disease process does not allow assessment of serial changes or the identification of global inhibition of cell proliferation induced by sirolimus, paclitaxel, or brachytherapy and thus is likely to result in fewer unwanted effects such as slow endothelialization and late stent thrombosis.

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Several limitations of our approach should be noted. First, tissue derived at 1 time point late in the disease process does not allow assessment of serial changes or the identification of early mechanisms in the disease process such as might be available from animal models.\textsuperscript{29} This may be why we saw no inflammatory signal for ISR in our ontology analysis. In addition, the impossibility of obtaining normal human control data might limit our ability to identify genes upregulated in both ISR and atherosclerosis. However, our intention was to identify the unique signature of ISR, and the best subtraction profile is clearly atherosclerosis rather than normal. Second, in network analysis, although publication bias is minimized by controlling for the size of the network and by combining semantic mining with experimental data, the former is limited by the available literature and by our choice of interaction verbs. The lack of more complex sentence processing means that a small number of sentences may be included that contain 2 terms and an interaction verb but do not imply a gene product–gene product relationship (estimates in pathways curated by hand suggest that this is between 5% and 15%). However, we believe that the unparalleled scale of analysis possible with computer processing, the benefit in including multiple cell types and models via the literature, and the ability of the technique to account for nontranscriptional regulation make up for these shortcomings and argue for the role of this integrative analysis alongside more established reductionist approaches.

**Conclusions**

In the present study, we have carried out an extensive assessment of the biology of human ISR. We confirmed previously reported findings from cellular microscopy studies and extended these into the molecular domain with nearly genome-wide transcriptional profiling. Ontology analysis of differentially regulated genes revealed a significantly more inflammatory profile for de novo atherosclerosis lesions than for ISR lesions. Network analysis confirmed this finding and identified subnetworks of highly connected genes that we hypothesize to be promising therapeutic targets. Finally, we tested our method by forcing it to predict whether sirolimus or paclitaxel would be the more effective agent in abrogating ISR and found that it successfully recapitulated the results of large-scale clinical trials. This is the first implementation of a network-based analysis of ISR and represents the most comprehensive survey of the disease process to date.

**Acknowledgments**

We would like to thank Lutz Buellesfeld for help in data collection and Eugene Yang for laying the groundwork for the study.

**Disclosures**

Angela Soito and Drs He and Simpson are employees of Foxhollow Technologies (Redwood City, Calif). Drs Tsalenko, Vailaya, and Kuchinsky are employees of Agilent Technologies (Palo Alto, Calif). Drs Ashley and Quertermous previously held consulting relationships with the Biologics Division of Foxhollow Technologies. Dr Grube is a stockholder of Foxhollow Technologies. The other authors report no conflicts.

**References**


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**CLINICAL PERSPECTIVE**

The present article investigates the biology of in-stent restenosis. Although drug-eluting stents have been highly successful in abrogating in-stent restenosis, they are not equally efficacious in all lesions and patients. In addition, questions remain over the incidence of in-stent thrombosis. In this study, we combined light microscopy with a state-of-the-art genomics tool, the microarray, to assess the gene expression of >20 000 genes in samples of atheroma removed from the coronary arteries of patients with coronary artery disease. We subtracted the gene expression profile of native lesions from those of in-stent disease to give the unique signature of in-stent restenosis. Using a new technique that parses the entire biomedical literature into networks using automated search, we overlaid this gene expression signature onto regulatory networks and scored each network for the average significance of its members. The highest-scoring networks were curated and tagged for further investigation. We predict that these networks will make excellent targets for future therapies against in-stent restenosis. To test the approach, we overlaid our gene expression signature on networks derived from the search terms “sirolimus” or “paclitaxel.” Our results, showing a mean significance score greater for the sirolimus network, recapitulated those of major clinical trials that show lower rates of late lumen loss for sirolimus-eluting stents. Future work will characterize the pathways identified and test their efficacy in abrogating in-stent restenosis.
Network Analysis of Human In-Stent Restenosis
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_Circulation_. 2006;114:2644-2654; originally published online December 4, 2006; doi: 10.1161/CIRCULATIONAHA.106.637025
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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