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# Prediction of Cell Type-Specific Gene Modules: Identification and Initial Characterization of a Core Set of Smooth Muscle-Specific Genes

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Genes that are expressed in the same subset of cells potentially constitute a module regulated by shared *cis*-regulatory elements and a distinct set of transcription factors. Identifying such units is an important entry point to the molecular study of cell differentiation. We developed a general method to classify cell type-specific genes from expressed sequence tag (EST) data, and we optimized it for identification of smooth muscle cell (SMC)-specific genes. Expression profiles were derived from the quantitative distribution of EST data in mouse, and genes were classified based on their profile similarity to known reference genes, in this case smooth muscle myosin heavy chain. A large majority (>90%) of known SMC-specific genes were identified, together with novel candidates. Extensive experimental validation confirmed SMC-specific expression of candidates, for example, lipoma preferred partner (LPP) and a novel SMC-specific putative monoamine oxidase, SMAO. Our method performed considerably better than other computational methods in an objective cross validation comparison. The total number of SMC-specific genes is estimated to be ~50.

[Supplemental material is available online at [www.genome.org](http://www.genome.org). A program package, *uni\_extract*, for extraction of data and data preparation, and a MATLAB program package, *QRISP*, for data transformation, probability estimation, cross validation, and visualization of data, is available at <http://cbz.gu.se/Lindahl/QRISP>. A gene expression pattern prediction server will be available at [www.qrisp.com](http://www.qrisp.com).]

A principal aim of modern developmental biology is to understand the differentiation of cell lineages in molecular detail. Regulation of cell type-specific genes is frequently used as a molecular model of cell differentiation. From a systems perspective, it is therefore interesting to identify complete sets of cell type-specific genes, and ask whether the same or different pathways regulate the correlated expression of such sets. The hierarchy of transcription factors that control expression of contractile proteins in skeletal muscle serves as one of the better understood molecular prototypes of mammalian cell lineage determination (Pownall et al. 2002), and comparative analyses of *cis*-regulatory elements indicate a common mechanism for several marker genes (Konig et al. 2002; Wasserman et al. 2000). Less is known about smooth muscle cells (SMCs). There has been considerable progress in mapping *cis*-regulatory elements required for expression of SMC-specific proteins (Mack et al. 2000; Chang et al. 2001; Manabe and Owens 2001a,b; Strobeck et al. 2001), but the presumed SMC-specific transcription factors that bind these elements have not been identified. Shared *cis*-regulatory elements (CArG boxes) in SMC marker promoters, in combination with correlated developmental onset of gene expression of some SMC markers, argue that there may be a modular regulation of the SMC genes. Still, the number of known SMC-specific genes is rather small, which limits the scope of molecular investigation, and SMC marker gene regulation has been studied from a single-gene perspective rather than by systematic side-by-side comparison. The present project was initiated to identify a near complete

set of SMC-specific genes to serve as an entry point for a system-oriented analysis of SMC-specific transcriptional regulation.

To identify SMC-specific genes is technically challenging. The broad tissue representation and the tight association between SMCs and other cell types make experimental screening difficult. SMC samples must be compared with a wide range of tissues, preferentially free of vasculature, to claim cell type specificity. In vitro experiments are of limited relevance because the cells are plastic and adopt a fibroblast appearance when cultured (Schwartz et al. 2000). The present work explores the potential of computational identification of SMC-specific genes from expressed sequence tag (EST) data as an alternative to experimental approaches.

Bioinformatic cell type specificity screens have been reported for a range of cells such as endothelial markers, colon cancer cells, and cardiac muscle (Wang et al. 2001; De Young et al. 2002; Huminiecki et al. 2002). Existing protocols for relating EST detection to cell type-specific expression of genes, such as keyword searches (Schuler 1997), and Digital Differential Display (Scheurle et al. 2000), rely on a well-characterized or obvious relationship between the expression pattern of interest and the EST libraries under study: Heart-specific genes are present in heart-derived libraries and absent from others, et cetera. The relationship between gene expression and library representation is less obvious in the SMC case, and more sophisticated analysis tools are therefore required.

An alternative approach for classification of EST data is to perform a multivariate analysis of expression profiles (a set of measurements of a gene's transcript abundance across a range of biological situations). Ewing and co-workers (Ewing et al. 1999; Ewing and Claverie 2000) have demonstrated the potential of unsupervised clustering of quantitative EST data in their analysis

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of the rice genome. We have optimized this approach and introduced a classification procedure that allows cell type-specific genes (e.g., SMC markers) to be discriminated from nonspecific genes. The key step in the optimization is an objective cross validation of alternative feature extraction methods (alternative ways to construct expression profiles) and alternative distance metrics (alternative ways to measure expression profile similarity).

The optimized procedure identified SMC-specific genes from mouse EST data with 99% specificity at 94% sensitivity. Extensive validation by *in situ* hybridization (ISH) confirmed that predictions of novel SMC marker genes were correct. Furthermore, the classification procedure outlined a module of ~50 SMC-specific genes in the genome, also in agreement with the ISH data.

In conclusion, the present work conceptually links profile-based EST analysis with classification/supervised learning and demonstrates its combined use in detecting modules of genes that are expressed in specific cell types with complex tissue representation. Classification, as opposed to cluster analysis, results in testable hypotheses on cell type specificity, which can be experimentally validated in a nonambiguous manner at success rates that can be estimated in advance.

## RESULTS

### Quantitative Expression Profiles Generated From Public EST Data Discriminate SMC-Specific Genes

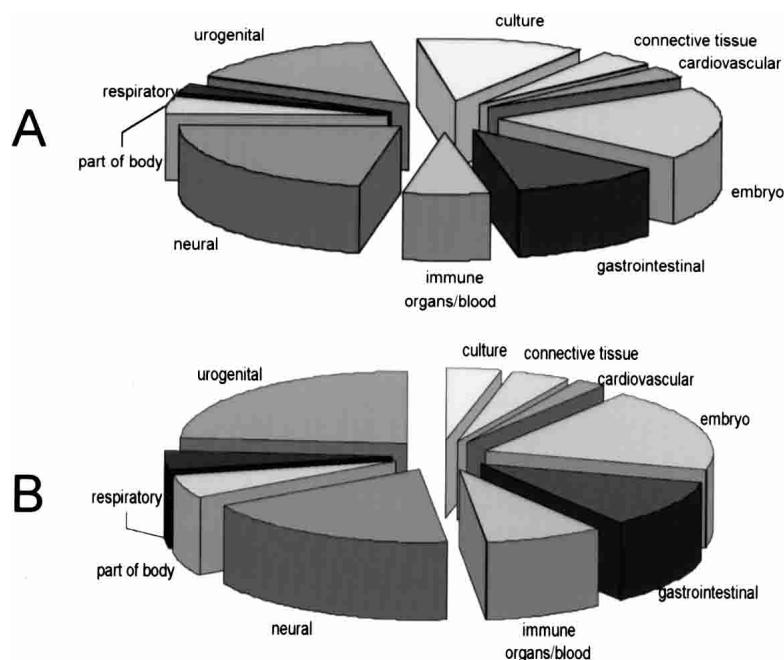
The mouse UniGene data contain 2.7 million ESTs from 639 different libraries (Fig. 1), and thus constitute an enormous source of transcript distribution information. SMCs are represented in practically all tissues, but in substantially different proportions. We therefore asked whether the differential presence of

SMCs between organs is sufficient to affect the library distribution of ESTs, and to generate an SMC-specific signature.

Expression profiles were constructed from the distribution of ESTs for each gene across the 639 libraries. The profiles of two known SMC markers, smooth muscle myosin heavy chain (SM-MHC) and leiomodulin 1, showed a high degree of correlation, indicating that profiles for SMC-specific genes might have characteristic features (Fig. 2). The correlation was primarily due to abundant transcript detection in urinary bladder and colon libraries. However, the degree of correlation between these two markers was clearly dependent on the type of expression profile that was constructed from the EST data (definitions of different data construction principles are given in Methods). Profiles that were derived from raw data (number of detections/library) and from transformed data (statistic estimate of gene representation/library) correlated very well. Profiles derived from frequency data (number of detections/library, normalized for library size) and binary data (presence/absence of gene/library, data not shown) did not show convincing correlation.

### Detection of SMC-Specific Gene Expression Can Be Defined as a Classification/Supervised Learning Problem

The pair-wise correlation between SMC marker profiles implied that SMC-specific genes might be detected by a classification (supervised learning) method. In such methods, user-provided examples ("training data") are used to predict the properties of novel objects. In this case, existing examples of known SMC markers, and examples of genes known *not* to be SMC markers can be used to estimate the probability for an undocumented gene to be SMC specific. We therefore developed a classifier that was optimized with respect to SMC marker identification. The classification has three components: (1) derivation of expression profiles from EST data, (2) computation of profile distances to a reference marker, and (3) estimation of probabilities (based on training data) for genes to have the same expression pattern as the reference marker.

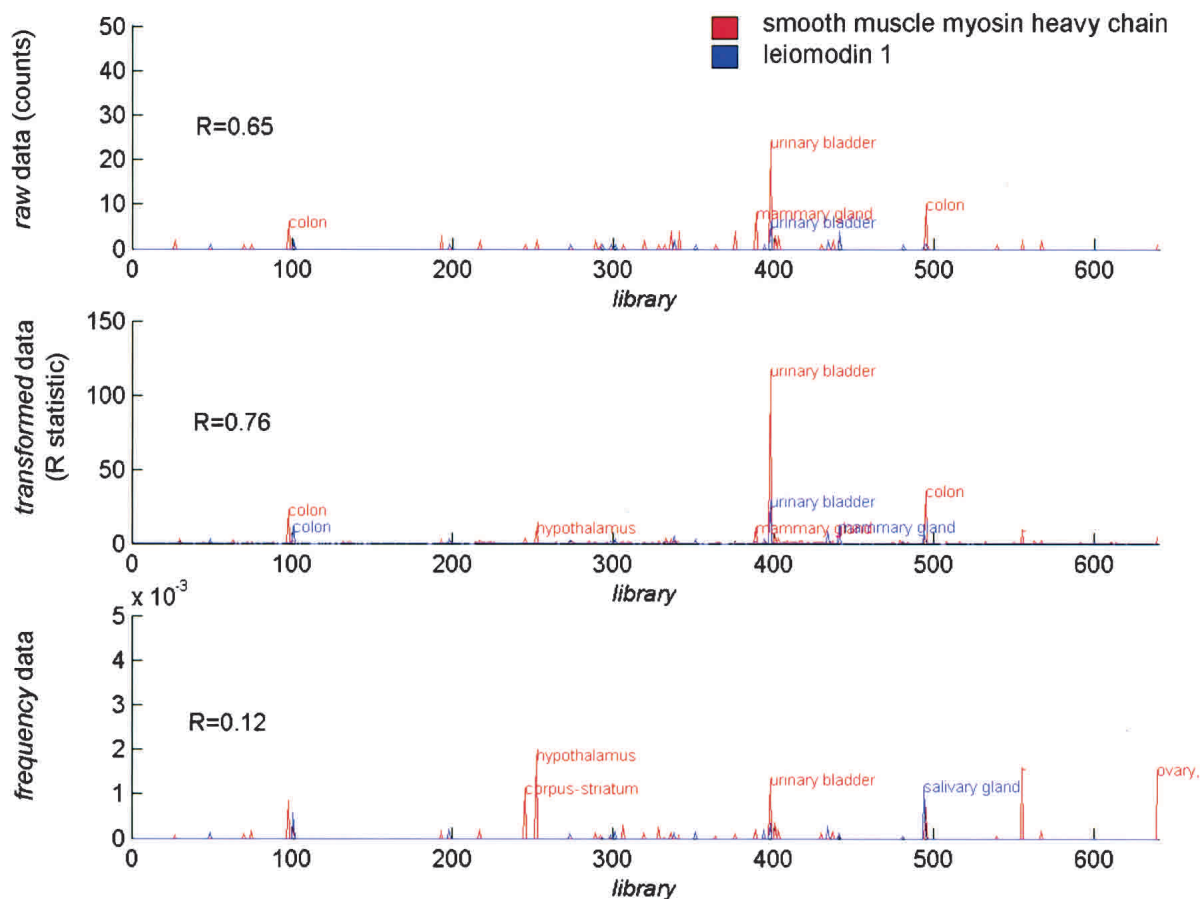


**Figure 1** Tissue sources represented in UniGene data. Based on tissue source information for the 639 mouse EST libraries, libraries were grouped into different organ systems (A). Embryo, whole embryo or part of embryo regardless of stage; part of body, combinations of adult organs or body parts; culture, cell culture, including stem cell libraries. Based on this grouping, the relative contribution from each tissue to the 2.7 million ESTs in the data was calculated (B).

### Systematic Evaluation of Alternative Classification Methods Established an Optimal Protocol for SMC Marker Prediction

To maximize sensitivity and specificity of discrimination, alternative technical parameters were systematically compared: The two parameters with the greatest impact on classification performance were (1) the type of profile and (2) the choice of distance metric.

The different profile types (raw, transformed, frequency, and binary) and the different distance metrics (permutation testing, Pearson's correlation coefficient, covariance, Fischer's test) are defined in Methods. In an informal evaluation, we ranked genes according to profile similarity to the SM-MHC gene and registered the ranks of 10 positive controls using different methods. This revealed a striking dependency on technical parameters (Fig. 3A). In a formal evaluation, we again used SM-MHC as the reference marker and estimated the probabilities for genes to be SMC specific using logistic regression fitted by unbiased training data as defined in Methods. This returned the estimated probability for different genes to be SMC specific, on the basis of alternative methods. In an optimal classification, the estimated probability for negative controls should be 0, and the estimated probability for positive controls (SMC mark-



**Figure 2** Examples of EST-derived expression profiles for two SMC-specific genes: *SM-MHC* (103 detections in UniGene, red curve) and *leiomodins 1* (28 detections in UniGene, blue curve). X-axis, The 639 mouse EST libraries in arbitrary order; Y-axis, EST-derived expression profiles according to definitions in Methods. For the *raw* and *transformed* profiles, the two SMC-specific genes have related profiles (correlation 0.65 and 0.76, respectively). Transcripts are primarily detected in certain urinary bladder and colon libraries. The two genes do not correlate in the frequency data, which also contain signals from unexpected organs such as the hypothalamus.

ers that were not included in fitting of the regression curve) should be 1.

Raw data and Pearson's correlation performed best, estimating the average SMC marker to 37% probability and the average nonmarker to a 1% probability (Fig. 3B,C). The use of binary profiles and the Fischer metric correspond to the hitherto best performing established method, GBA (Walker et al. 1999). However, this method only had an 8% probability for the average SMC marker and a 1% probability for nonmarkers, demonstrating that GBA should not be applied to SMC marker prediction. The informal evaluation (Fig. 3A) showed that, when using GBA, the top 3100 genes would need to be screened in order to detect ~90% of the known SMC-specific genes, compared to the top 280 genes when using raw data and Pearson's correlation. The formal sensitivity/specificity relationship for raw data and Pearson's correlation on a test set was 99% specificity at 94% sensitivity, and this method was used in all subsequent experiments. Predicted genes and probability estimates are shown in Table 1.

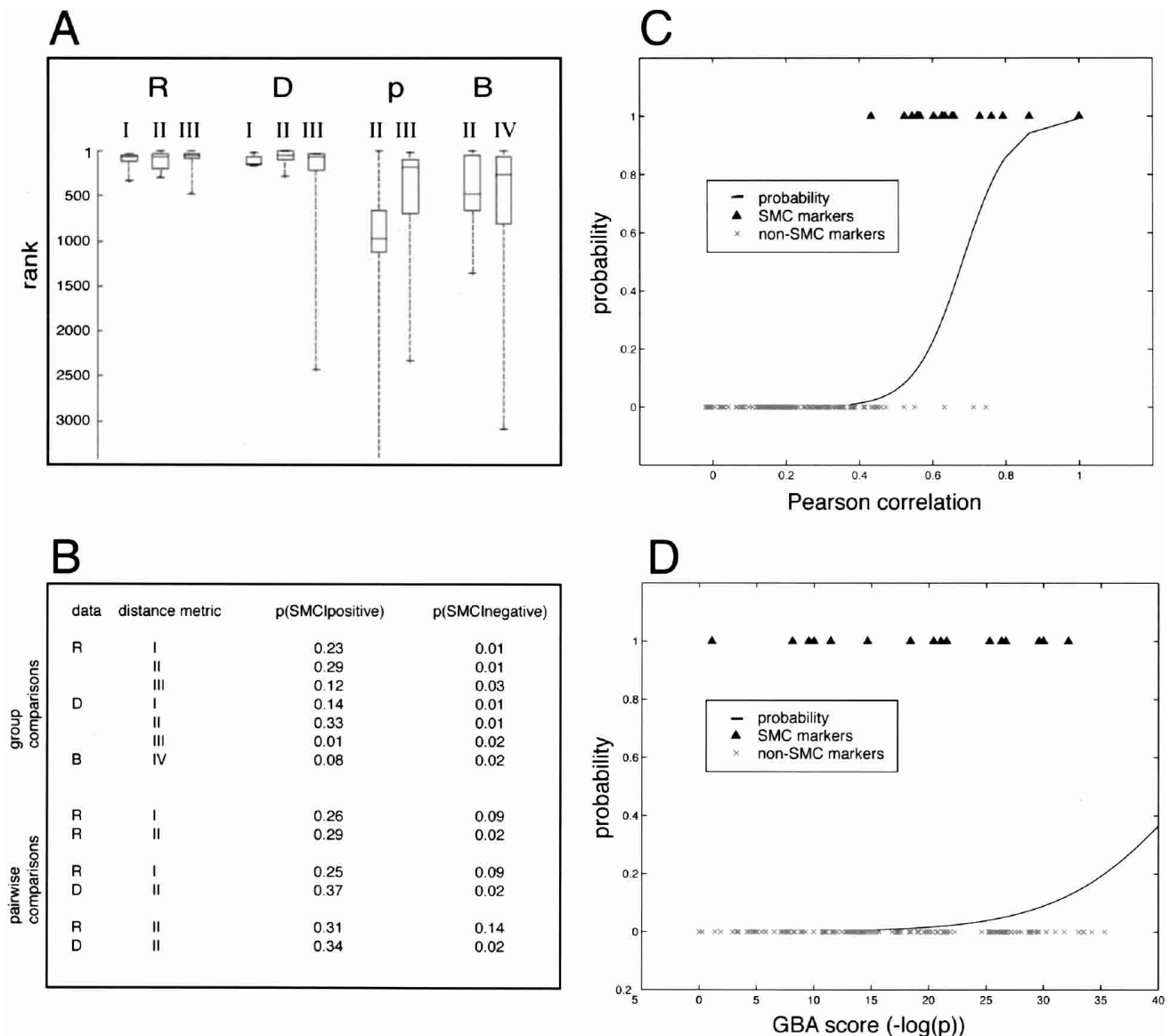
### In Situ Hybridization Confirmed SMC-Specific Expression of Candidate Genes and Revealed Systematic Patterns of Ectopic Expression

Forty-six genes with estimated probabilities between 1% and 77% were selected for further validation using ISH. The selection was fairly randomized with a slight bias toward abundant genes,

genes with "interesting features," and previously undocumented genes. The established SMC markers SM-MHC,  $\alpha$ -smooth muscle actin (ASMA), vinculin, and smoothelin were included to provide reference data. ISH was performed on mouse late embryonic (E17.5) sagittal sections, which allows for a wide range of organs to be screened on one slide. Embryonic expression is also relevant to validate a potential role in SMC development. ISH results were informative (presence of signal and low background) for 29 genes including the four controls. The result was used to classify the genes into three groups:

1. SMC marker group (four controls and five candidates). This group contained genes with an expression pattern highly concordant with expression of the SMC marker controls (Figs. 4, 5; Table 2). The SMC marker group displayed reoccurring patterns of ectopic expression in specific locations (Fig. 6).
2. SMC marker-related group (five candidates). This group contained genes with an SMC marker-concordant expression pattern but with additional atypical ectopic expression in a limited set of other locations (Fig. 7).
3. Genes with other expression patterns.

None of the known markers were strictly confined to SMCs but were to variable extents also expressed in cardiac and skeletal muscle (Figs. 4, 6). The markers were further expressed in some nonmuscle cells (SM-MHC, ASMA, and smoothelin stained sub-



**Figure 3** Systematic evaluation of alternative data types and distance metrics with respect to prediction of SMC-specific genes. R, transformed data; D, raw data; p, frequency data; B, binary data. Roman numerals indicate different distance metrics. I, Permutation; II, Pearson's correlation; III, covariance; IV, GBA (Walker et al. 1999). Euclidean distance was also evaluated, giving nonspecific results (not shown). For full definition of terms, see Methods. (A) Nearest neighbor searches were performed on all genes with at least five ESTs in UniGene ( $n = 29,812$ ). Genes were ranked according to their profile similarity to SM-MHC. Ranks for 10 SMC markers are displayed on the Y-axis as box blots. Boxes, Central 20<sup>th</sup> to 80<sup>th</sup> percentiles; whiskers, the full range of observations. (B) Alternative methods (combinations of data type and distance metric) were evaluated with a logistic regression model. This model was used to compute probabilities for genes to be SMC markers based on their profile distance to SM-MHC.  $P(\text{SMC}|\text{positive})$  denotes the model average probabilities for positive controls to be SMC markers, and  $P(\text{SMC}|\text{negative})$  denotes the corresponding probability for negative controls. Models were first compared group-wise using the full test set. This identified three preferred models (R-I, R-II, and D-II). These three models were then compared in a pair-wise fashion. To avoid bias, subsets of the reference gene set were used (see Methods). The preferred method was data = D, distance = II (Pearson's correlation), with expectations 0.37 for the markers and 0.02 for the nonmarkers. (C, D) Logistic regression curves for raw data/Pearson's correlation (C) and GBA (D). Triangles, Known SMC markers (probability = 1); crosses, genes with other expression pattern (probability = 0); curve, estimated relationship between a gene's correlation against SM-MHC and its probability to be an SMC marker.

mandibular gland epithelium; Fig. 5), ASMA and smoothelin were expressed in hair follicle dermal sheets, and ASMA finally was expressed in portions of the central nervous system (CNS). The CNS expression was not confined to vascular SMC.

The candidate genes were similarly expressed in all muscle lineages and in some additional cell types (Figs. 4, 5, 6; Table 2). Genes that recapitulated the SMC marker typical pattern of ectopic expression in submandibular glands, hair follicle dermal

sheaths, and regions in the CNS were classified as novel SMC markers (Fig. 5; Table 2). Genes with additional expression in atypical ectopic sites, for example, brown fat, bladder epithelium, and pancreas epithelium, were classified as SMC marker related (Fig. 7; Table 2).

Candidate genes that were classified to group (3) displayed the full spectrum of patterns from borderline cases of SMC-specific expression to ubiquitous expression. Approximately half



**Table 1.** Prediction of Smooth Muscle-Specific Genes

| Expression | Correlation | Probability | UniGene ID | Annotation                                   |
|------------|-------------|-------------|------------|--|
| 105        | 1.00        | 0.99        | Mm.3153    | <u>Myosin heavy chain 11, smooth muscle</u>  |
| 174        | 0.86        | 0.94        | Mm.16562   | <u>Actin gamma 2, smooth muscle, enteric</u> |
| 196        | 0.79        | 0.85        | Mm.2006    | <u>Transgelin/SM-22 alpha</u>                |
| 49         | 0.79        | 0.84        | Mm.43921   | RIKEN cDNA 4631426E05 gene                   |
| 68         | 0.78        | 0.83        | Mm.99349   | <u>Svs7</u>                                  |
| 33         | 0.77        | 0.80        | Mm.95705   | <u>Gamma-2 syntrophin</u>                    |
| 58         | 0.77        | 0.78        | Mm.3089    | ESTs   |
| 91         | 0.76        | 0.77        | Mm.31259   | <u>SMAO</u>                                  |
| 31         | 0.76        | 0.77        | Mm.99224   | ESTs   |
| 24         | 0.75        | 0.75        | Mm.46214   | <u>Uroplakin 3</u>                           |
| 57         | 0.74        | 0.70        | Mm.45481   | RIKEN cDNA 1110032A04 gene                   |
| 292        | 0.73        | 0.68        | Mm.6712    | <u>Desmin</u>                                |
| 26         | 0.72        | 0.66        | Mm.4351    | Arginine vasopressin receptor 1A             |
| 26         | 0.70        | 0.58        | Mm.100108  | RIKEN cDNA 5730414M22 gene                   |
| 31         | 0.68        | 0.50        | Mm.152763  | <u>Svs3</u>                                  |
| 32         | 0.66        | 0.43        | Mm.3787    | <u>Svs6</u>                                  |
| 344        | 0.66        | 0.42        | Mm.16537   | <u>Actin, alpha 2, smooth muscle, aorta</u>  |
| 31         | 0.65        | 0.40        | Mm.57221   | Homeobox B13                                 |
| 27         | 0.65        | 0.40        | Mm.180124  | <u>Leiomodin 1 (smooth muscle)</u>           |
| 41         | 0.64        | 0.34        | Mm.86380   | ESTs   |
| 29         | 0.63        | 0.33        | Mm.34359   | ESTs   |
| 26         | 0.63        | 0.32        | Mm.89958   | MRV integration site 1                       |
| 54         | 0.63        | 0.30        | Mm.25722   | <u>Purinergic receptor P2X</u>               |
| 76         | 0.62        | 0.27        | Mm.17807   | ESTs   |
| 22         | 0.61        | 0.25        | Mm.30933   | ESTs   |
| 99         | 0.60        | 0.24        | Mm.4356    | <u>Calponin 1</u>                            |
| 30         | 0.60        | 0.23        | Mm.88548   | ESTs   |
| 71         | 0.59        | 0.21        | Mm.24684   | ESTs   |
| 219        | 0.59        | 0.19        | Mm.10661   | Solute carrier family 2 member 4             |
| 222        | 0.58        | 0.19        | Mm.34060   | RIKEN cDNA 5830475I06 gene                   |
| 55         | 0.58        | 0.17        | Mm.45019   | Cancer related gene-liver 1                  |
| 286        | 0.58        | 0.17        | Mm.28278   | Caveolin, caveolae protein, 22 kD            |
| 50         | 0.57        | 0.17        | Mm.27165   | ESTs   |
| 134        | 0.57        | 0.16        | Mm.28649   | ESTs   |
| 189        | 0.57        | 0.16        | Mm.38877   | ESTs   |
| 60         | 0.57        | 0.15        | Mm.23983   | ESTs   |
| 81         | 0.57        | 0.15        | Mm.34171   | ESTs   |
| 43         | 0.57        | 0.15        | Mm.83243   | ESTs   |
| 49         | 0.57        | 0.15        | Mm.12966   | Plasma membrane associated protein, S3-12    |
| 284        | 0.57        | 0.15        | Mm.205997  | <u>LPP</u>                                   |
| 34         | 0.57        | 0.15        | Mm.34461   | ESTs   |
| 139        | 0.56        | 0.15        | Mm.22941   | RIKEN cDNA 4930435F02 gene                   |
| 307        | 0.56        | 0.14        | Mm.36850   | <u>Smoothelin</u>                            |
| 36         | 0.56        | 0.14        | Mm.85304   | Gob-5  |
| 147        | 0.56        | 0.14        | Mm.7342    | <u>PDZ and LIM domain 3</u>                  |
| 51         | 0.56        | 0.13        | Mm.314     | ESTs   |
| 31         | 0.56        | 0.13        | Mm.74658   | ESTs   |
| 76         | 0.56        | 0.13        | Mm.119265  | ESTs   |
| 466        | 0.55        | 0.12        | Mm.1987    | <u>Decorin</u>                               |
| 26         | 0.55        | 0.12        | Mm.55337   | ESTs   |
| 44         | 0.55        | 0.12        | Mm.87506   | ESTs   |
| 458        | 0.54        | 0.11        | Mm.196484  | <u>Cysteine rich protein 1</u>               |
| 94         | 0.54        | 0.11        | Mm.1237    | Peripheral myelin protein, 22 kD             |
| 51         | 0.54        | 0.11        | Mm.35290   | Expressed sequence A1552420                  |
| 111        | 0.54        | 0.11        | Mm.136207  | Expressed sequence AA986860                  |
| 66         | 0.54        | 0.10        | Mm.183030  | RIKEN cDNA 5430429D21 gene                   |
| 52         | 0.54        | 0.10        | Mm.44235   | HNF-3/forkhead homolog 1 like                |
| 65         | 0.53        | 0.10        | Mm.29953   | RIKEN cDNA 5430437E11 gene                   |
| 30         | 0.53        | 0.09        | Mm.38127   | Expressed sequence A1642000                  |
| 118        | 0.53        | 0.09        | Mm.27390   | ESTs   |
| 22         | 0.52        | 0.08        | Mm.112236  | ESTs   |
| 253        | 0.52        | 0.08        | Mm.36769   | <u>SLAP</u>                                  |
| 58         | 0.52        | 0.08        | Mm.31552   | ESTs   |
| 49         | 0.52        | 0.08        | Mm.23873   | ESTs   |
| ...        | ...         | ...         | ...        | ...  |
| ...        | ...         | ...         | [88 genes] | ...  |
| 39         | 0.43        | 0.02        | Mm.87553   | Protocadherin beta 17                        |

(continued)

of these genes showed some degree of SMC selectivity (data not shown). Three genes, finally, displayed restricted, but muscle unrelated, patterns.

The number of genes that experimentally qualified as being SMC markers was in agreement with the prediction

### The Degree of Ectopic Expression of Candidate Genes Is Recapitulated in the Profile's Distance to SM-MHC

Is there a correlation between the degree of SMC-specific expression and the distance to SM-MHC? ISH results were visualized by plotting SM-MHC profile similarity on the Y-axis versus gene abundance on the X-axis (Fig. 8). Gene abundance was defined as the logarithm of the total number of EST detections for a gene in UniGene. The ISH results revealed a logic relationship between the EST profile similarity to SM-MHC and the degree of SMC specificity. The expression in nonmuscle cells gradually increased with decreasing mathematical correlation to SM-MHC. The SMC marker group (1) had correlation ranging from 0.43–0.86, average 0.61. The SMC marker-related group (2) ranged between 0.52 and 0.57, average 0.54. Finally, the heterogeneous nonselective group (3) spread between 0.37 and 0.65, average 0.49. For genes with >50 detections, the separation between SMC markers and nonmarkers occurred in a correlation interval between 0.4 and 0.6. Only two low-abundance genes were experimentally validated; both were classified in the nonselective group (3). Data from the literature-based reference set were distributed in good accordance with the ISH data. However, the distribution also revealed a number of “false positives”, that is, non-SMC markers with high correlation. These were found, with rare exceptions, in the low-intensity range.

### The Mouse Genome Is Estimated to Encode ~50–60 SMC Markers

Probability estimation for genes to be SMC markers allows for a global estimate of the total number of SMC-specific genes (see Methods). We estimated probabilities by logistic regression that was fitted with the combined ISH and literature data (regression curve shown in Fig. 3C). Integrating probabilities gave a model estimate of 56 genes in the mouse genome that encode SMC markers (standard error 4.97). Of these, 34 are predicted to be among the top 161 genes with a profile correlation above 0.5, of which 12 are known markers. This indi-

**Table 1.** *Continued*

| Expression | Correlation | Probability | UniGene ID | Annotation                   |
|------------|-------------|-------------|------------|------------------------------|
| 25         | 0.43        | 0.02        | Mm.67539   | RIKEN cDNA 4933406D09 gene   |
| 25         | 0.43        | 0.02        | Mm.116986  | Secreted and transmembrane 1 |
| 216        | 0.43        | 0.02        | Mm.12842   | <u>Vinculin</u>              |

Top-ranking genes based on profile similarity to SM-MHC. Smooth muscle cell markers underlined. Likely false positives (the Svs and decorin) in italics. Expression, Number of detections in UniGene; correlation, Pearson's correlation in the raw data set (see Methods); probability, logistic regression probability estimate for a gene to be an SMC marker. For brevity, genes with fewer than 20 detections have been excluded. The logistic regression estimate of the total number of SMC markers in the list shown is 20.4; standard error 3.06.

cates that a majority of the unknown markers can be identified in a systematic screen of high-correlating genes.

### Quantitative EST Profiling Identified Structural and Regulatory Genes in Heart and Skeletal Muscle, Retina, and Lens, Demonstrating General Applicability

The method is stated in a general way and could theoretically be applied to any tissue or cell type. The only a priori information required is a marker gene that can be used as discriminator. Obviously, the lack of cell type-specific libraries reduces the resolution for some tissues: It is not possible to discriminate between closely associated cell types unless at least one library is enriched for one of the cells.

We profiled all genes with at least five UniGene detections ( $n = 29,812$ ) against a panel of genes with tissue-specific expression patterns (see Methods and Supplementary Material, available online at [www.genome.org](http://www.genome.org)). Examples of predictions based on skeletal muscle, heart muscle, photoreceptor, and lens markers are shown in Table 3A–D. Previously known markers for the respective cell type are consequently clustered at the top of the lists, thus confirming the accuracy. Fourteen of the top 19 genes with highest correlation to the cone-rod containing homeobox gene have a previous record of retina-specific expression. Compared with the SMC case, the Pearson's correlation coefficients in this case are higher, possibly indicating less complex expression patterns for these genes. Several well-documented key regulators of skeletal and cardiac muscle differentiation were identified among the structural genes.

## DISCUSSION

### Concordance Between Mathematical Predictions and Experimental Results

We have developed a new method for mathematical prediction of SMC-specific genes. Five SMC markers and five SMC marker-related genes with selective but not specific SMC expression were validated by ISH. The proportion of SMC markers versus non-markers in the experimentally evaluated gene set agrees well with the a priori estimated probabilities.

ISH offers a resolution and an organ overview that alternative methods such as Northern blot and RT-PCR cannot match. Still, classification of genes as SMC specific or not based on ISH is bound to be wrong sometimes. The screening was performed solely on embryonic sections and on the subset of organs that were simultaneously represented on single slides. Technical failures that result in artefact staining are sometimes mistaken for ubiquitous expression. We have consciously chosen to be conservative in judgments and to classify genes that have an SMC/muscle selective staining but high background staining as non-

muscle, which might lead to underestimation of specificity and to underestimation of the total number of SMC markers. We have also been careful not to claim technical failure unless it is obvious. The most common type of technical failure was complete lack of signal. Genes that are SMC specific only in certain splice isoforms (such as vinculin) may give ISH results that depend on the choice of probe. Our vinculin probe consisted of a 3' fragment of the vinculin mRNA, and should in theory not be able to differ between SMC-specific (metavinculin) and broadly expressed vinculin isoforms. Still, our ISH result for

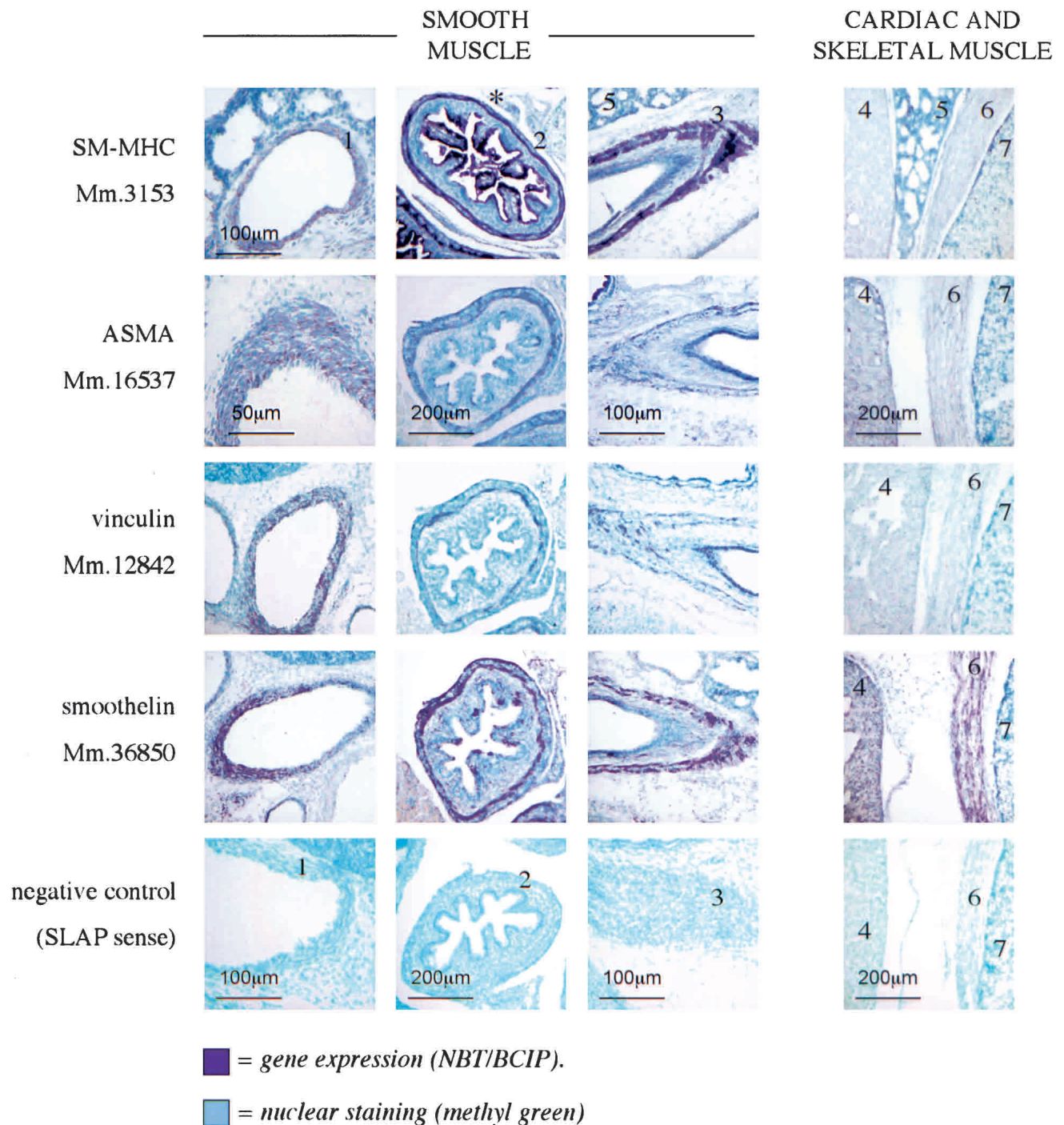
vinculin was highly SMC specific, possibly explained by a dominant contribution of metavinculin at this time point.

### Data Quantity and Library Diversity Are the Main Limiting Factors of Method Performance

The method is formulated in a general way to classify genes according to coexpression with a discriminator gene. This classification was used to identify cell type-specific markers, but theoretically it can be used to predict similarity to any discriminator gene expression pattern. The potential of the method in any particular case will depend on three factors: (1) the tissue sources underlying the data in relation to the expression pattern of interest, (2) the amount and quality of the data, and (3) the analysis method itself.

Predictions can only be made for cell types present in the data bank. Similarly, morphological resolution of the method depends on the presence of tissue- or cell type-specific libraries. Clearly, if two cell types are present in amounts that have the same ratio for all libraries, then the method cannot separate between genes specific for one or the other of these cell types. The efficiency of finding genes specific for cell type C depends on in how many libraries, and to what extent, the amount of C varies in relation to other cell types. It is, however, not necessary to have libraries containing only C. The sizes, that is, the total counts, of the libraries where C has a higher proportion cannot be too small. If they are, then the stochastic noise from the random selection of the sequenced tags will overwhelm any signal of differential expression. For the same reason, the C-specific genes that are to be detected cannot have too small expression rates in C compared with the sizes of the libraries where C has a higher proportion. Thus, the method is more likely to detect highly expressed C-specific genes than lowly expressed ones.

The current representation of tissues in mouse UniGene libraries (see Fig. 1) indicates that our prediction may be biased toward efficient detection of genes with expression in visceral and urogenital SMC and less efficient detection of genes that are preferentially expressed in vascular and pulmonary SMC. This indicates that the detection of SMC subtype-specific genes may be problematic with the current data set. However, the intention in this experiment has been to characterize a core set of general SMC markers that is present in all subtypes, and not to identify genes that are specifically expressed in certain subtypes. The UniGene data are a secondary data set derived from dbEST, a constantly growing public EST database, which indicates that resolution and tissue representation will improve over time. Other data sets that can be analyzed with the method in its current design are UniGene data for other species, or SAGE data. Potentially, data from several sources could be combined to enhance the power of the method. Improvements that could be attempted



**Figure 4** In situ hybridization (ISH) results for selected genes. Expression of control genes. 1, Vascular SMC; 2, intestinal SMC; 3, esophageal SMC; 4, cardiac muscle; 5, lung parenchyma; 6, diaphragm (skeletal muscle); 7, liver. A summary of ISH results is given in Table 2. They are also graphically depicted in Fig. 8. Intestinal lumen artefact staining is due to endogenous alkaline phosphatase activity (asterisk).

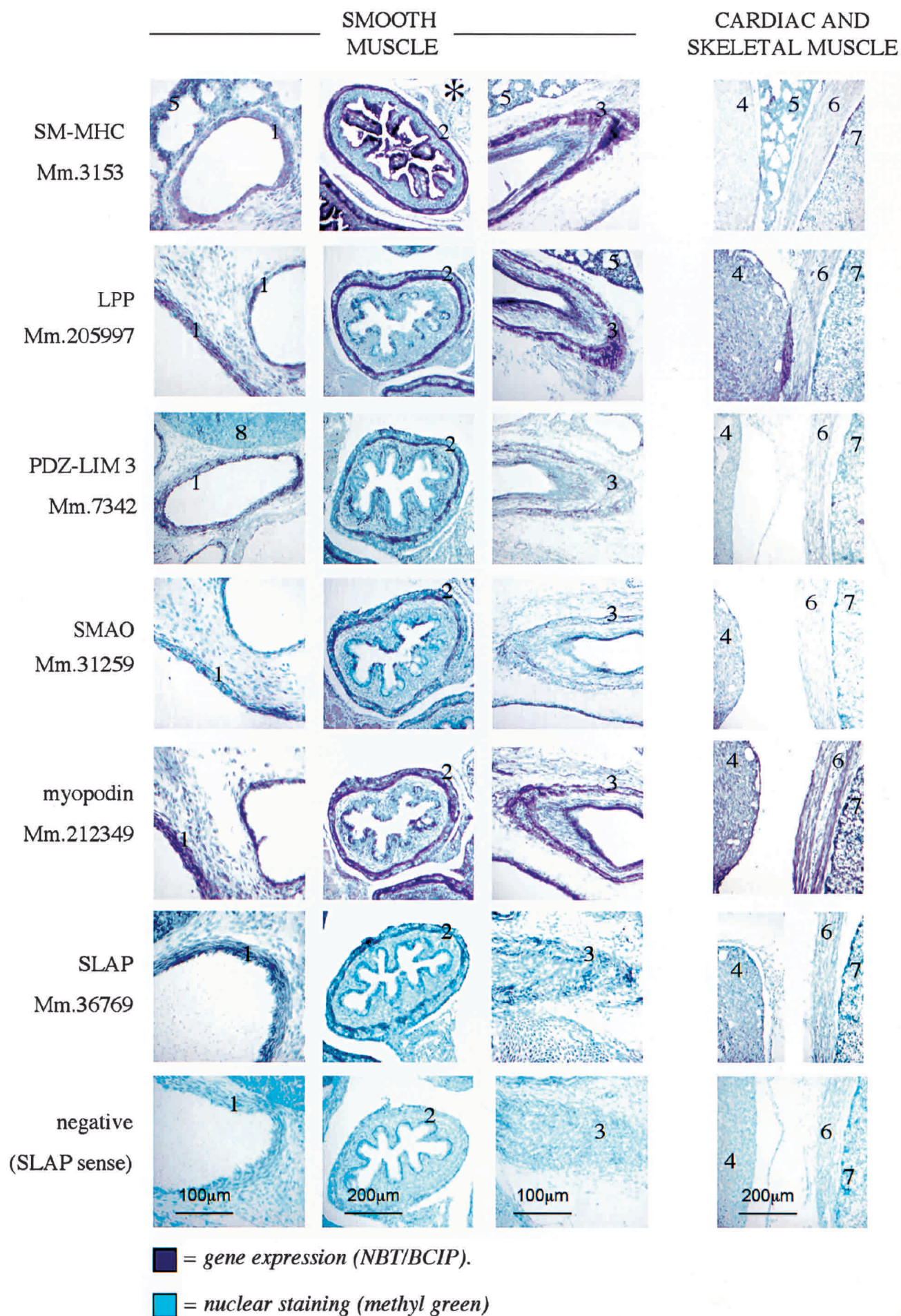
include mapping of SAGE data onto UniGene, addition of private-domain EST data to the public set, and cross comparing predictions versus microarray results. Further, it would be worth considering combinations of the method presented here with prediction methods based on other features, such as promoter sequences.

The libraries included are heterogeneous in terms of sampling depth and construction (sequencing direction, normaliza-

tion, et cetera), which is a potential source of systematic error. The annotation is another source of error: The one-to-one correspondence between genes and the EST clusters in UniGene can be questioned.

The predictive power using different data pretreatments and distance metrics was systematically evaluated. GBA, an established method for identification of expression modules, was clearly not performing well on genes with a more complex tissue





**Figure 5** (Legend on next page)

**Table 2.** Summary of In Situ Hybridization Results

| Expression | Correlation | Probability | UniGene ID | Annotation                       | IMAGE clone | In situ hybridization result |
|------------|-------------|-------------|------------|----------------------------------|-------------|------------------------------|
| 105        | 1.00        | 0.99        | Mm.3153    | Smooth muscle myosin heavy chain | 1547017     | SMC marker                   |
| 91         | 0.76        | 0.77        | Mm.31259   | SM-MAO                           | 1166836     | SMC marker                   |
| 344        | 0.66        | 0.42        | Mm.16537   | Alpha smooth muscle actin        | 1138966     | SMC marker                   |
| 284        | 0.57        | 0.15        | Mm.205997  | LPP                              | 2698658     | SMC marker                   |
| 307        | 0.56        | 0.14        | Mm.36850   | Smoothelin                       | 1162749     | SMC marker                   |
| 147        | 0.56        | 0.14        | Mm.7342    | PDZ and LIM domain 3             | 332852      | SMC marker                   |
| 29         | 0.56        | 0.13        | Mm.212349  | Myopodin                         | 1617930     | SMC marker                   |
| 253        | 0.52        | 0.08        | Mm.36769   | SLAP                             | 1025661     | SMC marker                   |
| 216        | 0.43        | 0.02        | Mm.12842   | Vinculin                         | 1096898     | SMC marker                   |
| 134        | 0.57        | 0.16        | Mm.28649   | Putative serine protease         | 5057524     | SMC marker-related           |
| 76         | 0.56        | 0.13        | Mm.119265  | No annotation                    | 347893      | SMC marker-related           |
| 94         | 0.54        | 0.11        | Mm.1237    | Pmp22                            | 1121305     | SMC marker-related           |
| 118        | 0.53        | 0.09        | Mm.27390   | Similar to human myoneurin       | 1195532     | SMC marker-related           |
| 58         | 0.52        | 0.08        | Mm.31552   | No annotation                    | 1365251     | SMC marker-related           |
| 9          | 0.75        | 0.75        | Mm.156846  | Putative myosin ATPase           | 1137251     | Nonselective                 |
| 10         | 0.65        | 0.40        | Mm.160140  | No annotation                    | 3412898     | Nonselective                 |
| 76         | 0.62        | 0.27        | Mm.17807   | No annotation                    | 1532956     | Nonselective                 |
| 6          | 0.58        | 0.18        | Mm.31071   | No annotation                    | 1052804     | Nonselective                 |
| 34         | 0.57        | 0.15        | Mm.34461   | Putative ATPase                  | 643914      | Nonselective                 |
| 51         | 0.56        | 0.13        | Mm.314     | No annotation                    | 1529589     | Nonselective                 |
| 66         | 0.54        | 0.10        | Mm.183030  | Weakly similar to ankyrin        | 1494583     | Nonselective                 |
| 77         | 0.50        | 0.06        | Mm.41387   | No annotation                    | 337790      | Nonselective                 |
| 229        | 0.48        | 0.04        | Mm.28406   | unc93 homolog B 86% similarity   | 1245118     | Nonselective                 |
| 352        | 0.48        | 0.04        | Mm.184314  | Putative dioxygenase             | 479719      | Nonselective                 |
| 69         | 0.48        | 0.04        | Mm.45173   | Msr2                             | 390123      | Nonselective                 |
| 101        | 0.47        | 0.04        | Mm.22588   | Similarity to human supervillin  | 949239      | Nonselective                 |
| 214        | 0.45        | 0.03        | Mm.10211   | Entpd5                           | 579841      | Nonselective                 |
| 117        | 0.41        | 0.02        | Mm.32011   | Kelch-related                    | 458907      | Nonselective                 |
| 349        | 0.38        | 0.01        | Mm.34315   | Similar to synembryn             | 5055713     | Nonselective                 |
| 410        | 0.37        | 0.01        | Mm.28793   | Snap25bp                         | 465348      | Nonselective                 |
| 31         | 0.65        | 0.40        | Mm.57221   | Hoxb13, homeobox B13             | 1026377     | Technical failure            |
| 222        | 0.58        | 0.19        | Mm.34060   | No annotation                    | 679316      | Technical failure            |
| 55         | 0.58        | 0.17        | Mm.45019   | No annotation                    | 2938912     | Technical failure            |
| 7          | 0.58        | 0.17        | Mm.139005  | No annotation                    | 2780347     | Technical failure            |
| 189        | 0.57        | 0.16        | Mm.38877   | No annotation                    | 4000106     | Technical failure            |
| 60         | 0.57        | 0.15        | Mm.23983   | Niban protein                    | 1344864     | Technical failure            |
| 139        | 0.56        | 0.15        | Mm.22941   | Putative methyltransferase       | 1449933     | Technical failure            |
| 7          | 0.51        | 0.07        | Mm.173134  | No annotation                    | 577206      | Technical failure            |
| 100        | 0.49        | 0.05        | Mm.196527  | Ankyring repeat domain protein   | 1514780     | Technical failure            |
| 26         | 0.48        | 0.04        | Mm.33974   | Unc gene homolog                 | 1617070     | Technical failure            |
| 121        | 0.44        | 0.02        | Mm.30693   | Basic Krüppel-like factor 4      | 3464873     | Technical failure            |
| 181        | 0.43        | 0.02        | Mm.25707   | No annotation                    | 1066724     | Technical failure            |
| 245        | 0.42        | 0.02        | Mm.29865   | Zinc finger domain-containing    | 425825      | Technical failure            |
| 80         | 0.41        | 0.02        | Mm.139238  | No annotation                    | 402913      | Technical failure            |
| 211        | 0.40        | 0.01        | Mm.28119   | Sgpl1                            | 1531669     | Technical failure            |
| 245        | 0.37        | 0.01        | Mm.31353   | Vps16 gene product               | 4317642     | Technical failure            |

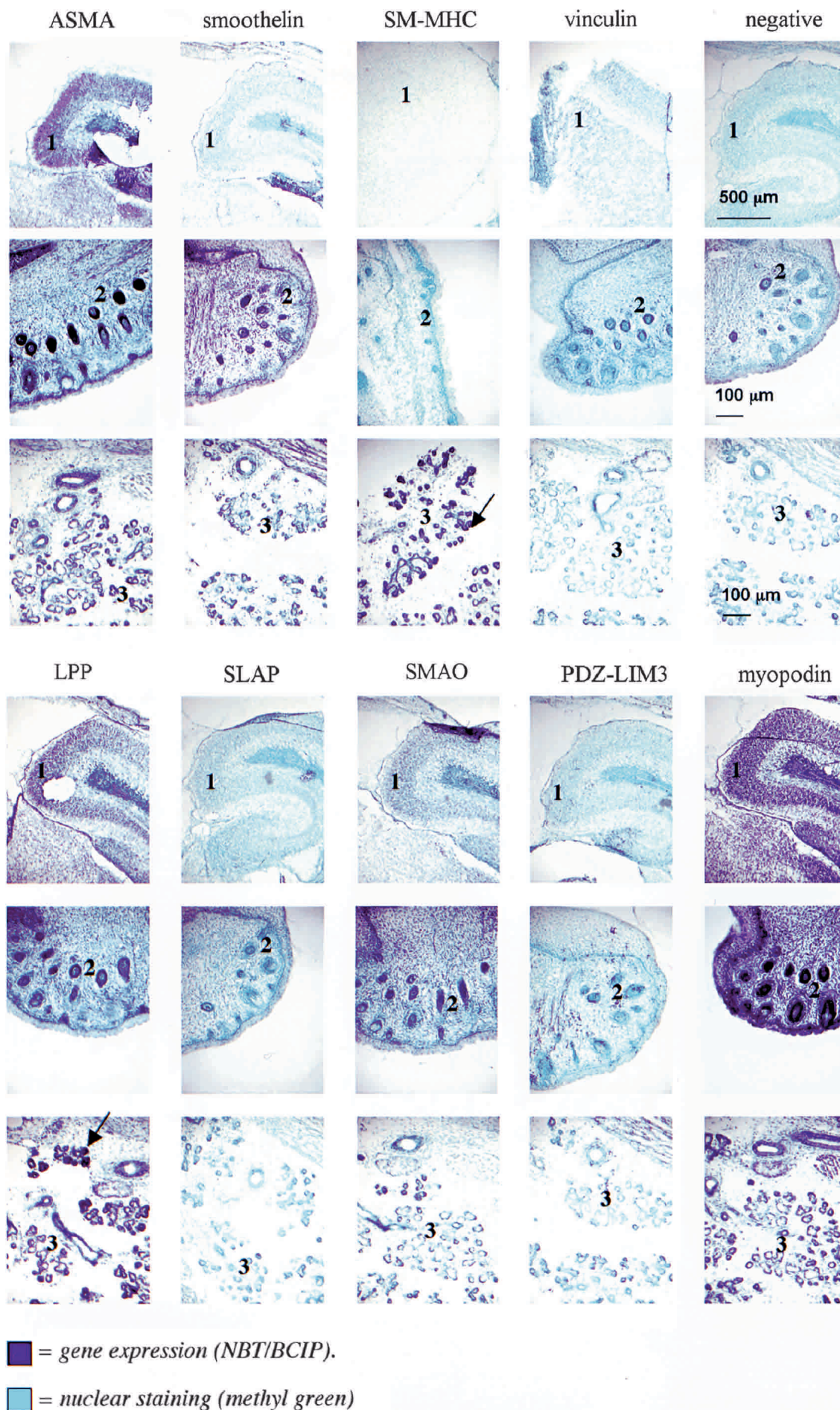
In situ hybridization validation of predicted SMC-specific genes. Forty-six EST clusters were chosen for validation by in situ hybridization in mouse E17.5 embryos. Genes were classified as SMC markers, SMC marker-related, nonselective, and technical failure, according to criteria described in Results. Expression, Number of detections in UniGene; correlation, Pearson's correlation with SM-MHC in the raw data set (see Methods); probability, logistic regression probability for a gene to be an SMC marker; IMAGE clone, probe template clone ID (see Methods). These results are visualized in Fig. 8. The logistic regression estimate of the total number of SMC markers in the list shown is 7.4; standard error 2.1.

distribution. Profiles generated from binary data and frequency data generally performed poorly. Similarly, established distance metrics such as Euclidean distance and covariance were less suitable. With the current data set, we saw no clear benefit in using statistical transformation or permutation testing (Fig. 3) in comparison with simpler approaches such as Pearson's correlation. The fact that the size of an EST library ( $10^2$ – $10^5$  tags) is small in relation to the often minute frequencies at which individual genes are represented in the transcriptome ( $10^{-6}$  to  $10^{-1}$ ) indicates that expression levels can only be crudely estimated from EST data.

Genes differ markedly in their absolute expression level. Rare transcripts have a low representation in EST libraries, are less well detected in assays such as ISH and Northern blotting, and give weak signals with a large experimental error on fluorescence-based chip assays such as cDNA microarrays. As with any other method, the EST screening procedure is likely to be more error prone with rare transcripts. Our definition of expression level is based on the sum of UniGene detections. In the analysis presented earlier, we anticipated that any cluster with fewer than five (arbitrary limit) members was unlikely to give reliable results. Filtering the data gave 29,812 clusters with five or more mem-

**Figure 5** ISH data for genes that were classified as SMC markers. Vascular SMC; 2, intestinal SMC; 3, esophageal SMC; 4, cardiac muscle; 5, lung parenchyma; 6, diaphragm (skeletal muscle); 7, liver. Intestinal lumen artefact staining is due to endogenous alkaline phosphatase activity (asterisk).





**Figure 6** (Legend on next page)

bers, which is in parity to the estimated 30,000–40,000 genes in mammals.

### Lipoma Preferred Partner and Smooth Muscle Monoamine Oxidase: Two SMC-Specific Genes With Potential Regulatory Functions

On the basis of protein sequence and previously published data, two of the newly detected SMC markers stand out as interesting genes with potential regulative functions in SMC biology: lipoma preferred partner (LPP) and smooth muscle monoamine oxidase (SMAO).

#### LPP

LPP belongs to the zyxin protein family (Petit et al. 1996). The family members zyxin, Trip6, ajuba, LIMD1, and LPP have similar structures with a proline-rich domain at the N terminus and three successive LIM domains at the C terminus. LPP and other members of the family are shuttled between focal adhesions and the nucleus in vitro (Petit et al. 2000; Petit et al. 2003). The significance of the nuclear localization is not clear, but LPP strongly enhances transcription in a Gal4-reporter system. Previously published adult human Northern blot data (Petit et al. 1996) are compatible with smooth muscle-specific expression, although the connection was not made. The name LPP was motivated by frequent fusion between LPP and the HMGIC gene in lipomas, indicating a possible role in tumor development (Petit et al. 1996).

Recently, Chang et al. (2003) showed that CRP-1 and 2 act as adapters between serum response factor (SRF) and GATA4/6 to form a transcription factor complex that induces expression of SMC-specific genes such as SM-MHC in vitro. CRP-1 and 2 also shuttles between the nucleus and the cytoplasm and has been shown to bind zyxin (Sadler et al. 1992). Hypothetically, LPP is the endogenous binding partner of the SRF-CRP-GATA complex in SMC and a coregulator of SMC-specific transcription. Targeted disruption of the zyxin gene in mice did not generate any obvious phenotypic changes, possibly due to function overlap between zyxin family members (Hoffman et al. 2003).

#### SMAO

The SMAO gene is previously undocumented, and belongs to a single-gene family. Domain annotation identified a C-terminal flavin-containing monoamine oxidase (MAO) domain. A Psi-BLAST search against the National Center for Biotechnology Information (NCBI) protein database showed that the predicted MAO domain matches MAO domains of numerous other proteins at 20%–35% sequence identity, including mammalian MAO-A and MAO-B proteins. Mouse SMAO has unique rat and human orthologs, with ~90% global protein sequence identity. On the basis of domain information, it is possible that SMAO functions as a smooth muscle-specific or smooth muscle-selective MAO enzyme. As such, it may contribute to the MAO activity registered in, for example, aortic SMCs (Jaakkola et al. 1999). MAO activity in vascular SMC has been implicated as a potential source of advanced glycosylation end products (Mathys et al. 2002). SMC specificity, a potential for pharmacological manipulation, and coupling to medically relevant processes motivate further studies of this protein.

### Potential Roles in SMC Biology for Previously Described Muscle Proteins and for Pmp22

An unexpected finding was that peripheral myelin protein 22 (Pmp22) showed strong selective muscular expression, with ex-

pression in specific SMC subsets at E17.5 (Fig. 7). This gene has previously been reported to be expressed at nonneural locations during embryogenesis, indicating a function not restricted to neural processes (Baechner et al. 1995). Another member of the Pmp22 family, epithelial membrane protein 3 (Emp3) is predicted as one of the extreme top correlators of fast skeletal muscle myosin light chain (Table 3B). An intriguing hypothesis is that different members of the Pmp22 family of proteins participate in muscle processes, in a subtype-dependent manner.

The ISH data demonstrated SMC/muscle selectivity for a number of genes with existing documentation in cardiac and skeletal muscle: sarcolemmal-associated protein, PDZ-LIM3 or alpha actinin-associated LIM protein, and myopodin (Fig. 5). These may represent a subset of muscular proteins that participate in SMC processes. Furthermore, the gene matching the cluster Mm. 119265 (Fig. 7) is homologous to the actin-bundling genes palladin and myopalladin, indicating a role in actin bundling.

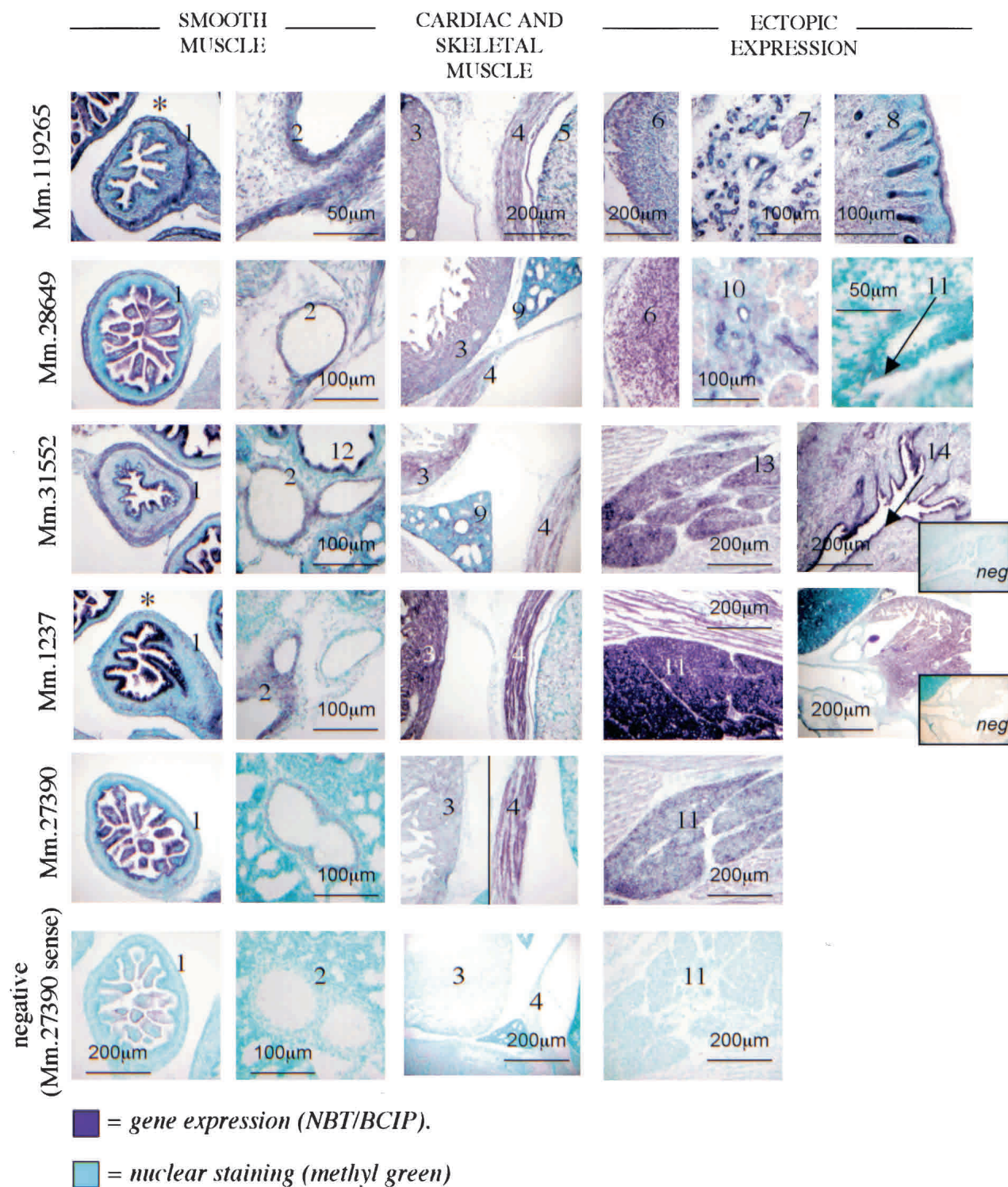
### Can Upstream Developmental Regulators Be Extrapolated From a Core Set of Cell Type-Specific Genes?

This work was initiated to identify a core set of SMC-specific genes. The expression of these genes is controlled, directly or indirectly, by transcription factors involved in the developmental regulation of the cell phenotype. A critical question is whether upstream regulators coexpress with downstream (e.g., structural) genes to such a degree that they may be detected by EST screening.

A majority of documented SMC-specific genes depend on SRF for their expression (Mack et al. 2000; Chang et al. 2001; Manabe and Owens 2001a,b; Strobeck et al. 2001). SRF is not SMC specific but is expressed more widely in other muscle types and in other tissues (Chai and Tarnawski 2002). In the SM-MHC-based search, SRF appears on rank 333, slightly below the top 1 percentile, which is compatible with an expression pattern that is related to SM-MHC. The idea that upstream developmental regulators may be found among top-ranking genes is supported by the presence of several well-documented regulators in the top 1 percentile of skeletal- and heart muscle-specific genes: The transcription factors myogenin, MyoD1, and MRF4 are key regulators of skeletal muscle induction in vivo (Pownall et al. 2002). A search based on skeletal muscle myosin light chain (UniGene ID Mm.1000) detected these regulators at ranks 248, 43, and 19, respectively. A fourth factor, Myf5 (Pownall et al. 2002), was not represented in the data analyzed. A search based on cardiac myosin heavy chain (UniGene ID Mm.3776) identified the following developmental regulators at top positions: the mouse *tinman* homolog Nkx2.5 (Cripps and Olson 2002) at rank 27, the *Xin* (Wang et al. 1999) at rank 50, and the transcription factor myocardin (Wang et al. 2001) at rank 80. Other factors indicated in the same process, such as MEF2C, dHand, eHand, Gata4, and Gata6 (Cripps and Olson 2002) do not appear in top-ranking positions in this search, possibly indicating a more general expression pattern or a temporally restricted heart-specific expression. For SMC biology, the obvious future prospect of formalized EST screening is to pinpoint SMC-specific candidate genes with potential developmental and regulatory functions. However, SMC-specific expression does not necessarily require SMC-specific regulators, but may result from combinatorial interac-

**Figure 6** Ectopic expression of SMC markers in the CNS, hair follicles, and the submandibular gland. Expression in the submandibular gland epithelium was confined to the distal tubular system (arrows). 1: CNS, 2: hair follicle dermal sheaths, 3: submandibular glands.

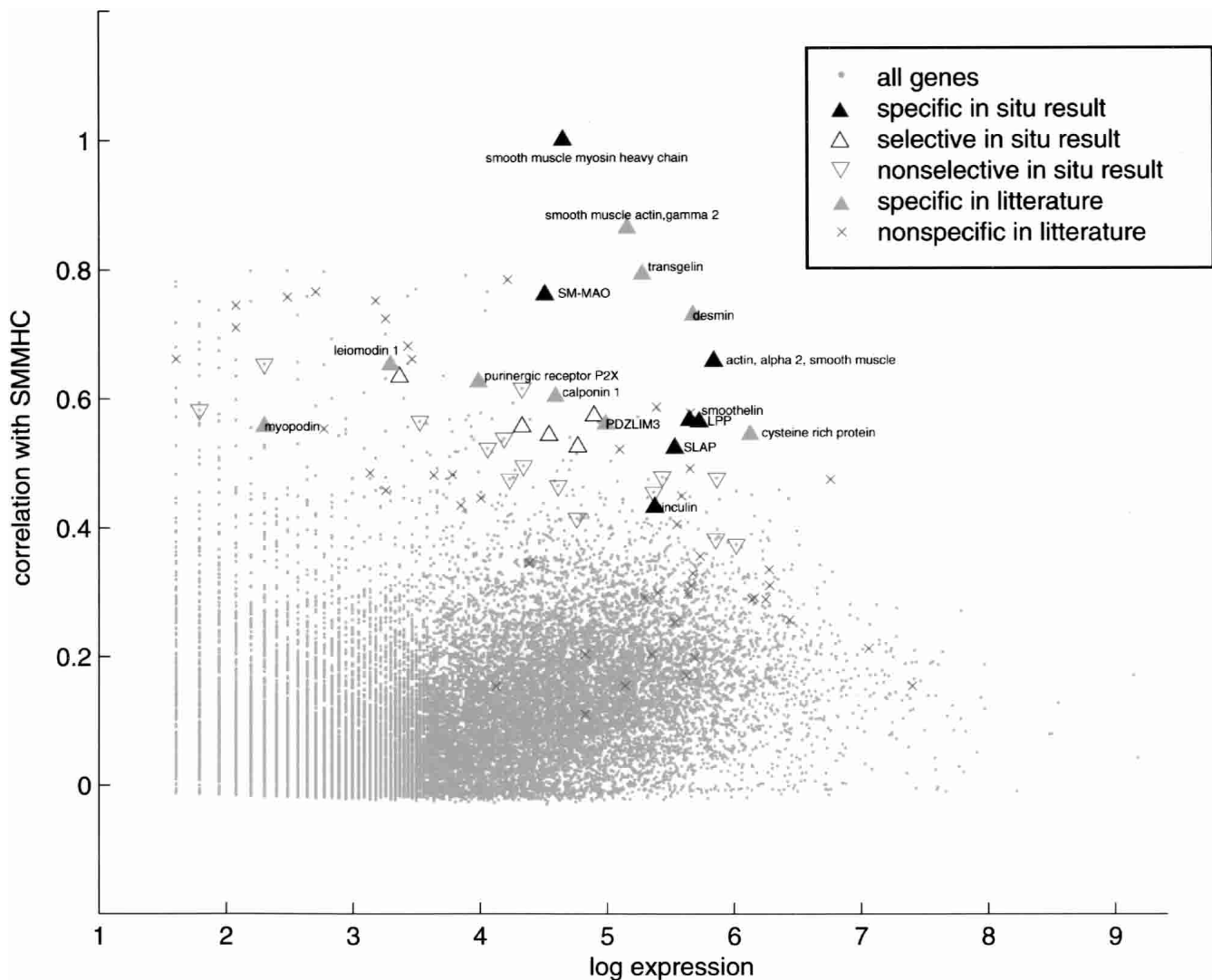




**Figure 7** ISH data for SMC marker-related genes. Expression was prominent in muscle cells but was also seen in a range of ectopic sites. 1, Intestinal SMC; 2, vascular SMC; 3, cardiac muscle; 4, diaphragm (skeletal muscle); 5, liver parenchyma; 6, CNS; 7, submandibular gland; 8, hair follicle (general mesenchymal expression seen); 9, lung parenchyma; 10, pancreas; 11, liver; 12, airway; 13, brown adipose tissue; 14, urinary bladder. Intestinal lumen artefact staining is due to endogenous alkaline phosphatase activity (asterisk).

tions between factors with less restricted expression patterns. Nkx3-1 and SRF form a complex that has been shown to regulate the smooth muscle  $\gamma$ -actin gene in vitro (Carson et al. 2000). Similarly, the previously mentioned SRF-CRP1/2-GATA4/6 com-

plex induced the expression of several SMC-specific genes including SM-MHC in vitro (Chang et al. 2003). Our EST screening is optimized to predict genes with correlating expression boundaries and may not identify less restricted regulators.



**Figure 8** ISH results in relation to profile similarity to SM-MHC (Y-axis) and to expression level (X-axis). Expression level is defined as the logarithm of the number of UniGene detections for a gene, and profile similarity is defined as Pearson's correlation in the raw data set. Gray dots, All 29,812 genes with at least five UniGene detections; dark upward-pointing triangles, ISH-detected SMC markers; open upward-pointing triangles, ISH-detected SMC marker-related genes; downward-pointing triangles, genes with a nonselective expression pattern in the ISH experiment; grey upward-pointing triangles, SMC markers from the literature; crosses, nonselective genes from the literature.

### Support for Extended SMC Properties of Myoepithelial Cells

Starfish-shaped cells in the outer layers of exocrine gland epithelium share features of both epithelium and SMCs. In the mammary gland, these myoepithelial cells have been shown to express several SMC markers including ASMA, SM-MHC, calponin, and h-caldesmon (Lazard et al. 1993). Myoepithelial cells in other exocrine glands have been shown to express ASMA and calponin (Ogawa et al. 1999). The myoepithelial cells have ultrastructural features that are typical of SMC and they have been shown to contract (Redman 1994). Our data show that, in addition to the previously known genes ASMA and SM-MHC, several SMC-specific genes are expressed in the submandibular gland epithelium, including LPP, SMAO, and myopodin. The resolution of our data is not sufficient to exactly define the spatial location of the SMC marker expressing cells within the epithelium. It seems likely that they are synonymous with the previously described myoepithelial cells, or progenitors of these cells. The growing number of SMC-specific genes that are expressed in

myoepithelium indicates that this cell type should be more consciously considered in studies of SMC differentiation and in studies of regulation of SMC-specific genes.

### Ectopic Expression in Nonmuscle Cells Indicates Coregulation of SMC-Specific Genes?

Our ISH experiments showed frequent expression of the known markers in nonmuscle tissues such as the submandibular gland epithelium. Interestingly, the new markers were detected in the same ectopic locations. Coordinated expression of a group of genes is either a result of shared upstream regulators, or an effect of convergent evolution where unique combinations of *cis*-regulatory elements in the individual genes result in overlapping expression. Large numbers of coexpressed genes and a complex expression pattern decreases the probability of convergent evolution being the explanatory factor. SMC-specific genes are expressed in selective subsets of cells originating from all three germ layers: neural crest-derived vascular SMC, mesoderm-

**Table 3.** Prediction of Specific Expression in Other Tissues

| Expression                                      | Correlation | ID        | Annotation  |
|---|-------------|-----------|---|
| A. Prediction of cardiac-specific genes         |             |           |   |
| 270   | 1.00        | Mm.3776   | Myosin heavy chain, cardiac muscle, adult                     |
| 127   | 0.82        | Mm.155714 | Myosin, heavy polypeptide 7, cardiac muscle, beta             |
| 59  | 0.82        | Mm.10728  | Myosin binding protein C, cardiac                             |
| 389   | 0.82        | Mm.686    | Actin, alpha, cardiac   |
| 137   | 0.72        | Mm.1529   | Myosin light chain, phosphorylatable, cardiac ventricles      |
| 58  | 0.71        | Mm.20271  | Fibrillin 2   |
| 47  | 0.69        | Mm.19961  | ESTs, highly similar to atrial natriuretic factor             |
| 201   | 0.67        | Mm.632    | Troponin T2, cardiac  |
| 51  | 0.66        | Mm.46514  | Myosin light chain, regulatory A                              |
| 11  | 0.66        | Mm.89854  | A disintegrin and metalloprotease domain 11                   |
| 6   | 0.66        | Mm.171448 | ESTs, weakly similar to mouse forkhead protein O1A            |
| 10  | 0.64        | Mm.207070 | <i>Mus musculus</i> heart alpha-kinase mRNA, partial cds      |
| 43  | 0.64        | Mm.89727  | CD8beta opposite strand                                       |
| 5   | 0.60        | Mm.33561  | ESTs  |
| 5   | 0.60        | Mm.125583 | ESTs  |
| 5   | 0.60        | Mm.208235 | ESTs  |
| 6   | 0.59        | Mm.158756 | RIKEN cDNA 2610019F11 gene                                    |
| 8   | 0.59        | Mm.27037  | ESTs  |
| 52  | 0.59        | Mm.17235  | Cysteine-rich protein 3                                       |
| 109   | 0.58        | Mm.43     | Myosin light chain, alkali, cardiac atria                     |
| 136   | 0.57        | Mm.6375   | Glycogenin 1  |
| 7   | 0.57        | Mm.89976  | Zinc finger, imprinted 1                                      |
| 11  | 0.57        | Mm.210460 | ESTs  |
| 5   | 0.56        | Mm.209027 | ESTs  |
| 12  | 0.56        | Mm.218070 | ESTs  |
| 36  | 0.56        | Mm.4211   | Solute carrier family 8 (sodium/calcium exchanger), member 1  |
| 7   | 0.56        | Mm.41974  | Nkx2.5/tinman homolog   |
| 8   | 0.55        | Mm.116789 | Repressor of GATA   |
| 5   | 0.55        | Mm.160735 | ESTs  |
| 5   | 0.55        | Mm.189024 | ESTs  |
| B. Prediction of skeletal muscle-specific genes |             |           |   |
| 341   | 1.00        | Mm.1000   | Myosin light chain, alkali, fast skeletal muscle              |
| 31  | 0.80        | Mm.57093  | Calcium channel, voltage-dependent, gamma subunit 1           |
| 18  | 0.77        | Mm.18125  | Expressed sequence AI118095                                   |
| 28  | 0.74        | Mm.33171  | Ankyrin repeat and SOCs box-containing protein 5              |
| 51  | 0.74        | Mm.140604 | RIKEN cDNA 1110002H13 gene                                    |
| 428   | 0.71        | Mm.214950 | Actin, alpha 1, skeletal muscle                               |
| 124   | 0.71        | Mm.29358  | RIKEN cDNA 2700055K07 gene                                    |
| 156   | 0.71        | Mm.29994  | Enolase 3, beta muscle  |
| 260   | 0.70        | Mm.14526  | Myosin light chain, phosphorylatable, fast skeletal muscle    |
| 121   | 0.69        | Mm.14546  | Troponin T3, skeletal, fast                                   |
| 107   | 0.69        | Mm.20829  | Epithelial membrane protein 3                                 |
| 254   | 0.68        | Mm.39469  | Troponin I, skeletal, fast 2                                  |
| 43  | 0.68        | Mm.46232  | Integrin beta 1 binding protein 2                             |
| 38  | 0.67        | Mm.4583   | Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle) |
| 41  | 0.67        | Mm.144259 | RIKEN cDNA 2310050C09 gene                                    |
| 52  | 0.65        | Mm.31099  | ADP-ribosyltransferase 1                                      |
| 152   | 0.65        | Mm.36900  | Troponin I, skeletal, slow 1                                  |
| 135   | 0.65        | Mm.1583   | Lymphocyte antigen 6 complex, locus C                         |
| 12  | 0.65        | Mm.11     | MRF4  |
| 14  | 0.64        | Mm.58214  | RIKEN cDNA 2310002L13 gene                                    |
| 78  | 0.64        | Mm.712    | Troponin C, cardiac/slow skeletal                             |
| 22  | 0.64        | Mm.140151 | Small proline-rich protein 1B                                 |
| 51  | 0.63        | Mm.125614 | Myosin binding protein H                                      |
| 21  | 0.63        | Mm.2810   | Cholinergic receptor, nicotinic, gamma polypeptide            |
| 27  | 0.63        | Mm.45734  | RIKEN cDNA 2310024D23 gene                                    |
| 110   | 0.63        | Mm.878    | Lymphocyte antigen 6 complex, locus D                         |
| 24  | 0.63        | Mm.45137  | RIKEN cDNA 2310032K21 gene                                    |
| 34  | 0.62        | Mm.10194  | Myomesin 2  |
| 272   | 0.62        | Mm.43831  | Lectin, galactose binding, soluble 1                          |
| 19  | 0.61        | Mm.36668  | RIKEN cDNA 1110008K04 gene                                    |
| C. Prediction of retina-specific genes          |             |           |   |
| 67  | 1.0         | Mm.8008   | Cone-rod homeobox containing gene                             |
| 92  | 1.0         | Mm.1205   | Rod photoreceptor 1   |
| 94  | 1.0         | Mm.1372   | Phosphodiesterase 6B, cGMP, rod receptor, beta                |
| 37  | 1.0         | Mm.78749  | Retinitis pigmentosa 1 homolog (human)                        |
| 418   | 1.0         | Mm.69061  | Alpha transducin  |
| 23  | 1.0         | Mm.206228 | ESTs  |

(continued)



**Table 3.** *Continued*

| Expression                           | Correlation | ID        | Annotation   |
|--------------------------------------|-------------|-----------|--|
| 62                                   | 1.0         | Mm.59151  | ESTs   |
| 830                                  | 1.0         | Mm.2965   | Similar to rhodopsin                                   |
| 85                                   | 1.0         | Mm.151562 | <u>Similar to retinol-binding protein</u>              |
| 6                                    | 1.0         | Mm.172488 | ESTs   |
| 38                                   | 1.0         | Mm.194050 | ESTs   |
| 31                                   | 1.0         | Mm.1370   | Phosphodiesterase 6A, cGMP-specific, rod, alpha        |
| 15                                   | 1.0         | Mm.23793  | Cyclic nucleotide gated channel, cGMP gated            |
| 38                                   | 1.0         | Mm.42102  | <u>Tubby like protein 1</u>                            |
| 38                                   | 1.0         | Mm.41982  | Retinoschisis 1 homolog (human)                        |
| 23                                   | 1.0         | Mm.39200  | Phosphodiesterase 6G, cGMP-specific, rod, gamma        |
| 114                                  | 1.0         | Mm.1276   | <u>Retinal S-antigen</u>                               |
| 29                                   | 1.0         | Mm.95707  | RIKEN cDNA A930007I01 gene                             |
| 82                                   | 1.0         | Mm.679    | <u>Rod outer segment membrane protein 1</u>            |
| 59                                   | 1.0         | Mm.20422  | <u>Neural retina leucine zipper gene</u>               |
| D. Prediction of lens-specific genes |             |           |  |
| 135                                  | 1.0         | Mm.1228   | <u>Crystallin, alpha A</u>                             |
| 25                                   | 0.9         | Mm.30374  | <u>Crystalline, gamma C</u>                            |
| 7                                    | 0.9         | Mm.130559 | ESTs   |
| 16                                   | 0.9         | Mm.127184 | ESTs, weakly similar to A39757 beta-crystallin         |
| 64                                   | 0.9         | Mm.22830  | <u>Crystalline, beta A1</u>                            |
| 9                                    | 0.9         | Mm.22861  | ESTs   |
| 5                                    | 0.9         | Mm.215166 | ESTs   |
| 7                                    | 0.9         | Mm.209940 | ESTs   |
| 45                                   | 0.9         | Mm.89477  | <u>Crystalline, gamma F</u>                            |
| 48                                   | 0.9         | Mm.31625  | Major intrinsic protein of eye lens fiber              |
| 13                                   | 0.9         | Mm.95578  | ESTs   |
| 17                                   | 0.9         | Mm.86656  | <u>Crystalline, beta A2</u>                            |
| 22                                   | 0.9         | Mm.168942 | BR3B   |
| 7                                    | 0.9         | Mm.180528 | ESTs   |
| 25                                   | 0.9         | Mm.138345 | ESTs   |
| 93                                   | 0.9         | Mm.127171 | <u>Beaded filament structural protein in lens-CP94</u> |
| 5                                    | 0.9         | Mm.209953 | ESTs   |
| 26                                   | 0.9         | Mm.215250 | Solute carrier family 7                                |
| 9                                    | 0.9         | Mm.137178 | RIKEN cDNA E130202I16 gene                             |
| 11                                   | 0.9         | Mm.138333 | Ras p21 GTPase   |

Nearest neighbor searches were performed with four different tissue-specific genes: cardiac-specific myosin heavy chain (A), skeletal muscle myosin light chain (B), the retina-specific cone-rod homeobox (C), and the lens-specific genes crystallin alpha A (D). Top 30 genes are shown in A and B. Top 20 genes are shown in C and D. On the basis of literature validation, genes specifically expressed in each tissue were identified (underlined in each list). Expression, Number of detections in UniGene.

derived visceral and vascular SMC, and endoderm-derived sub-mandibular epithelium. This is a highly complex expression pattern, which gives indirect support for the idea of a coregulated SMC expression module.

## Perspective

This work establishes mathematical prediction as a mode for expanding the molecular repertoire of SMC biology. We have identified a number of novel markers and estimated the total number of SMC-specific genes to be ~50. We anticipate that the remainder of these genes can be identified by this methodology. Further, comparative studies of *cis*-regulatory elements, for example, by phylogenetic footprinting approaches, in the now expanded core set of SMC markers may provide more information of the gene networks regulating the differentiation of SMCs.

## METHODS

### 1. Bioinformatics and Statistics

#### Data Preparation

The aim of the data preparation step is to produce a tag sampling data matrix on the form  $D_{ij}$  = number of detections of gene  $i$  in library  $j$ . Mouse UniGene data were downloaded from the NCBI server (<ftp://ftp.ncbi.nih.gov>). Other potential sources of tag

sampling data include SAGEDb (Lash et al. 2000) and BodyMAP (Hishiki et al. 2000). EST counts were extracted from the UniGene flat files using software that can be downloaded from <http://cbz.gu.se/Lindahl/EST>. In the UniGene-derived matrix  $D_{ij}$ , rows correspond to UniGene clusters and columns to cDNA libraries. Subsequent analysis was performed with programs written in the MATLAB (MathWorks, Inc.) language. Data were filtered to exclude UniGene clusters with fewer than five (arbitrary limit) members, leaving 29,812 clusters.

#### EST-Derived Expression Data

The EST data preparation resulted in a matrix on the form  $D_{ij}$  = number of tag detections of gene  $i$  in library  $j$ . In this matrix, the sum of  $D_{.j} = D_{1j} + D_{2j} + \dots + D_{Nj}$  corresponds to the size of library  $j$ , whereas the sum of  $D_{i.} = D_{i1} + D_{i2} + \dots + D_{iM}$  is the number of detections of gene  $i$  in the data ( $N$  = number of genes, and  $M$  = number of libraries).  $D_{i.}$  can be seen as a global measure of the abundance of gene  $i$ . The analysis is based on interpreting tag counts as expression levels of genes. The naïve use of the tag counts in  $D_{ij}$  as the expression signal is termed “raw data” (Fig. 2). A variety of the raw data matrix,  $B_{ij}$ , termed “binary data” was calculated using  $B_{ij} = 0$  if  $D_{ij} = 0$ ,  $B_{ij} = 1$  if  $D_{ij} > 0$ .

Viewing the number of detections in a certain library as fixed, the distribution of detection events across different genes can be modeled statistically. A test statistic  $R_{ij}$ , termed *transformed data* (Fig. 2), was formulated: for each cluster  $C$  and each library  $L$ , the R statistic tests whether the proportion of the sequences in



L that are in C could be the same as the corresponding proportion for all other libraries combined. More precisely, a generalized likelihood ratio test (Rice 1995) is preformed. Let,  $P_L$ , respectively,  $P_o$ , be the probability for a sequence in L, respectively in all other libraries, to be in C. Let  $D_{LC}$  and  $D_{Lo}$ , respectively  $D_{oC}$  and  $D_{oo}$ , be the observed counts of sequences in C and out of C for L, respectively, for all other libraries. Assuming binomial distributions for the data, the maximum likelihood estimates become

$$\hat{p}_L = \hat{p}_o = \frac{D_{LC} + D_{oC}}{D_{LC} + D_{Lo} + D_{oC} + D_{oo}}$$

under the null hypothesis

$$P_L = P_o,$$

and

$$\tilde{p}_L = \frac{D_{LC}}{D_{LC} + D_{Lo}}, \tilde{p}_o = \frac{D_{oC}}{D_{oC} + D_{oo}}$$

otherwise. The generalized likelihood ratio test statistic becomes

$$R = -2 \log \frac{\hat{p}_L^{D_{LC}} (1 - \hat{p}_L)^{D_{Lo}} \hat{p}_o^{D_{oC}} (1 - \hat{p}_o)^{D_{oo}}}{\tilde{p}_L^{D_{LC}} (1 - \tilde{p}_L)^{D_{Lo}} \tilde{p}_o^{D_{oC}} (1 - \tilde{p}_o)^{D_{oo}}},$$

with an approximate  $\chi^2$  distribution with one degree of freedom under the null hypothesis.

We also calculated the proportion of transcripts for each gene in a library,  $p_{ij} = D_{ij}/D_{\cdot j}$ , termed "frequency data."

#### Distance Metrics

The distance metrics evaluated were Pearson's correlation coefficient and covariance. For the binary data, standard correlation and the use of Fischer's exact test (Rice 1995) were applied, the latter corresponding to a previously published method, GBA (Walker et al. 1999). We also constructed an alternative metric based on pair-wise permutation testing, and interpretation of the  $P$  value as coexpression. More precisely, for each pair of profiles, the correlation between them with the libraries permuted in one of them is computed for a number of permutations. The rank of the actual correlation in this set of numbers can be interpreted as a  $P$  value for the hypothesis that the profiles are enriched in unrelated libraries. We interpreted this  $P$  value as a distance between the profiles.

#### Nearest Neighbor Search and Annotation

Let  $v(i)$  be the expression profile for gene  $i$  (i.e., row  $i$  in one of the data matrices  $D_{ij}$ ,  $B_{ij}$ ,  $R_{ij}$ , and  $p_{ij}$ , discussed earlier). Given one single training example,  $b$ , and a distance metric, the distance between  $v(b)$  and  $v(1), v(2), \dots, v(N)$  was calculated ( $N$  = the number of genes). Genes were then sorted according to distance and ranked. From the top-ranking genes downward, genes were annotated against the Celera Discovery System and public databases. Genes with sufficient published expression information were classified as SMC specific or not.

#### SMC-Specific Reference Gene Set

The following set of smooth muscle-specific genes was used as a reference set in the evaluation of data treatments and distance metrics below: alpha smooth muscle actin (ASMA, Acta2), smooth muscle myosin heavy chain (SM-MHC, Myh11), gamma smooth muscle actin (Actg2), desmin (Des), leiomodulin 1 (Lmod1), SM-22-alpha (transgelin, Tagln), smoothelin (Smtn), calponin 1 (Cnn1), cysteine-rich protein 1 (Crp1), and vinculin (Vcl).

#### Logistic Regression

Logistic regression was used to model the probability of two genes sharing the same expression pattern, based on profile similarity. The model assumes a linear relationship between profile

similarity  $x$  and probability  $p$  on the form  $\ln[p/(1-p)] = a + bx$ , where  $a$  and  $b$  are the parameters estimated from "training data" (i.e., examples of SMC markers and nonmarkers; see following). Our implementation is based on the MATLAB statistics toolbox. The model returns  $p_i$  = the probability for gene  $i$  to be an SMC marker based on the profile distance ( $x_i$ ) to SM-MHC. Parameters of the model were fitted by literature data, or literature data in combination with in situ results.

The expectation of the number of genes with the same expression pattern in a specific set, for example, the top-ranking 200 genes, is given by calculating the sum of  $p_i$ 's in that set. The standard error for the expectation is estimated as the square root of the sum over all  $p_i(1-p_i)$  in the set.

#### Logistic Regression Training Data

In order to compare alternative methods with respect to classifying genes as SMC markers or non-SMC markers in such a way as to not benefit one specific method, and because it was unfeasible to annotate the full set of genes, the following reference set of genes was constructed. The 1% top-ranking ( $n = 289$ ) genes were identified using seven alternative search methods: 1–6, nearest neighbor searching against SM-MHC in the raw ( $D_{ij}$ ) and transformed ( $R_{ij}$ ) data matrices using Pearson's correlation coefficient, permutation testing, and covariance as distance metrics. 7, nearest neighbor searching against SM-MHC in the binary data ( $B_{ij}$ ) matrix using GBA. Merging lists and removing redundancies produced a list of 975 genes. Annotation of this list resulted in a test set with 14 positive examples (SMC markers) and 134 negative examples (non-SMC markers, that is, genes documented as having another expression pattern). In order to include a reasonable number of negative examples for the remaining 29,812–975 genes, a random subset of 200 genes from this set was annotated. Nineteen negative examples and 0 positive examples were found. Based on this,  $(29,812 - 975 - 200) \times 19/200 = 2720$  genes were randomly selected and used as additional negative examples.

#### Comparison of Classification Performance

Using distance data from the seven different methods described earlier, the literature reference data were analyzed by logistic regression. In a simulation that was repeated 50 times, the 14 positive and 134 negative reference genes were split into a training set (75% of the genes in each group) and a test set (remaining 25% in each group). The logistic model was then fitted using the training data plus additional random negative examples (in order to avoid overestimation of probabilities due to insufficient annotation, see earlier). Model predictions of the probabilities for genes in the test set to be SMC markers were recorded, and expectations (mean values) calculated. To further compare methods in a pair-wise fashion, subsets of reference genes present in the top 1% list of either method were used.

## 2. Experimental Validation

#### DIG-RNA Probe Preparation

Clones in the vector pT3T7-PAC representing candidate UniGene clusters were purchased from Research Genetics, Inc. Plasmid midi-preparations were done according to Sambrook et al. (1989) and plasmids were linearized by restriction digestion with *NotI* (sense probes), and *EcoRI* (antisense probes). One microgram of linearized plasmid was purified with a DNA cleanup kit (Zymo Research, Inc). Digoxigenin-labeled cRNA probes were made with in vitro transcription using T3 (antisense probes) and T7 (sense negative control probes) RNA polymerase (Roche).

#### In Situ Hybridization

E17.5 mouse embryos were fixed overnight in 4% PFA in PBS, and further incubated in 30% sucrose in PBS. Embryos were mounted in Optimal Cutting Temperature compound (Sakura) and stored at  $-80^\circ\text{C}$ . Fourteen-micrometer cryosections on SuperFrost plus slides were heated to  $55^\circ\text{C}$  for 60 sec and stored at  $-80^\circ\text{C}$ . incubated at  $37^\circ\text{C}$  for 30 min, and washed 5 min each in

PBS, PBS 0.1% tween-20, and PBS. Sections were permeabilized with proteinase K 5 µg/mL in TE at 30°C for 30 min, and fixed for 5 min in 4%PFA in PBS. Further steps were performed as described at [www.roche-applied-science.com/prod\\_inf/manuals/InSitu/pdf/ISH\\_181-188.pdf](http://www.roche-applied-science.com/prod_inf/manuals/InSitu/pdf/ISH_181-188.pdf).

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