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Integrated Membrane Protein Analysis of Mature and Embryonic Stem Cell-derived Smooth Muscle Cells Using a Novel Combination of CyDye/Biotin Labeling*

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Cultivated vascular smooth muscle cells (SMCs) were surface-labeled with CyDyes followed by biotinylation. After enrichment on avidin columns, proteins were separated on large format gradient gels by SDS-PAGE. A comparison between CyDye-tagged and non-tagged gel bands revealed a substantial increase of protein identifications from membrane, membrane-associated, and extracellular matrix proteins with a corresponding reduction in co-purified intracellular proteins. Notably the majority of identified proteins were involved in cellular adhesion processes. To demonstrate the quantitative potential of this platform, we performed a comparison between mature and embryonic stem cell-derived smooth muscle cells (esSMCs) and identified the membrane proteins E-cadherin, integrin α6, and CD98 (4F2) to be significantly up-regulated in esSMCs suggesting that SMCs derived from embryonic stem cells maintain characteristics of their embryonic stem cell origin. This was subsequently confirmed by RT-PCR: despite expressing a panel of smooth muscle markers (calponin, *Sm22*, and aortic smooth muscle actin), esSMCs remained positive for markers of stem cell pluripotency (*Oct4*, *Nanog*, and *Rex1*). In summary, we describe a novel strategy for the profiling of cell membrane proteins. The procedure combines DIGE technology with biotin/avidin labeling to discriminate membrane and membrane-associated proteins from intracellular contaminants by fluorescence tagging and permits semiquantitative differential expression analysis of membrane proteins. ***Molecular & Cellular Proteomics 6:1788–1797, 2007.***

The proteins embedded in the plasma membrane are involved in fundamental cellular processes including signal reception/transduction, adhesion, solute transport, and interaction with cytoskeleton and extracellular matrix. The importance of this subset is expressed in the fact that about 50% of commercially available drugs target plasma membrane proteins (1). Therefore, differential membrane proteome analysis is a highly desired approach for the discovery of new diagnostic and therapeutic molecules. Additionally from a biology standpoint, this protein class may provide missing links for a basic understanding of cellular function. For these reasons, plasma membrane protein analysis has become an area of substantial interest for many proteomics investigations.

A major drawback in proteomics analysis of membrane proteins is the low abundance of these proteins. Because membrane proteins are dramatically underrepresented compared with cytosolic proteins, prior to analysis, most studies have attempted to reduce complexity and thus enrich the membrane fractions. To achieve this, several techniques including density gradient centrifugation (2), detergent-based fractionation (3), and isolation by biotin/avidin labeling (4) have been used. However, differential protein expression analysis is problematic due to experimental variability during the extraction procedure and contamination by high abundance proteins, which hampers the identification and quantification of membrane proteins present in low copy numbers. So far, quantitative membrane differential analysis has mainly been achieved by MS-based proteomics using HysTag (5) or ICAT (6) labeling, but precious instrument time is wasted on co-purified contaminant proteins in the membrane preparation. Hence there is a need for a method to restrict analysis to proteins of interest.

In the current report, we propose a new gel-based proteomics approach capable of restricting analysis to membrane proteins while facilitating differential analysis. Briefly surface proteins are first labeled using DIGE fluors adapted from Mayrhofer *et al.* (7), then targeted using biotin labeling, enriched on avidin affinity columns, and finally separated using common SDS-PAGE. In this method, the biotin/avidin step reduces sample complexity while the additional fluorescence tag allows membrane and membrane-associated proteins to be readily distinguished from co-purifying contaminant proteins. We confirm the applicability of the platform by using the approach 1) for membrane protein profiling of vascular

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smooth muscle cells (SMCs)[1] and 2) differential expression analysis of membrane proteins from embryonic stem cell-derived smooth muscle cells (esSMCs) and mature aortic SMCs.

MATERIALS AND METHODS

Antibodies—The following antibodies were used: α-tubulin (ab7750, 1:100, Abcam), heat shock protein 90 (sc-7947, 1:300, Santa Cruz Biotechnology), α-actin (sc-1616, 1:1,000, Santa Cruz Biotechnology), lactate dehydrogenase (ab7639, 1:1,000, Abcam), integrin β1 (sc-6622, 1:100, Santa Cruz Biotechnology), neural cell adhesion molecule (RDI-NCAM13abm, 1:500, Fitzgerald), transforming growth factor β receptor II (sc-400, 1:100, Santa Cruz Biotechnology), CD98 (sc-7094, 1:200, Santa Cruz Biotechnology), integrin α6 (sc-6597, 1:200, Santa Cruz Biotechnology), and E-cadherin (ab40772, 1:500, Abcam).

Smooth Muscle Cell Culture—Vascular SMCs from C57BL mice were cultivated from aortas as described by Hu *et al.* (8). SMCs were cultured on gelatin-coated flasks in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 15% fetal calf serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and passaged by treatment with 0.05% trypsin, 0.02% EDTA solution. The purity of SMCs was routinely confirmed by immunostaining with antibodies against aortic smooth muscle actin. Experiments were conducted on SMCs achieving subconfluence at passages 15–25.

Embryonic Stem Cell-derived Smooth Muscle Cell (esSMC) and SMC Culture—esSMCs were obtained *in vitro* using a method recently established in our laboratory (9). esSMCs were cultivated on gelatin-coated flasks in basic differentiation medium: α-minimal essential medium (Invitrogen) supplemented with 10% FCS (Invitrogen), 50 μM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and passaged by treatment with 0.05% trypsin, 0.02% EDTA solution. Experiments were conducted on esSMCs achieving subconfluence at passages 15–25. To allow differential expression analysis of esSMCs with SMCs, the latter cells were also cultivated in basic differentiation medium.

Biotin Targeting of Plasma Membrane Proteins—For each labeling reaction, cells were grown in two 150-mm dishes until reaching 90% confluency. Approximately 4×10^7 cells (2×10^7 cells/dish) were quickly washed twice with 8 ml of ice-cold PBS^{2+} (0.1 M phosphate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.2). The sulfo-NHS-SS-biotin (12 mg) was dissolved in 48 ml of ice-cold PBS^{2+} (approximately 0.25 mg/ml), and 10 ml was added to each dish. Next dishes were placed on a rocking platform and agitated for 30 min at 4 °C to ensure even coverage of the cells with the labeling solution. To stop the reaction, 500 μl of quenching solution (PBS^{2+} with 100 mM glycine) was added to each dish. Gently cells were scraped into solution, and the contents of all two dishes were transferred to a 50-ml conical tube and centrifuged at 500 × *g* for 3 min, and the supernatant was discarded. The cell pellet was washed twice using 5 ml of TBS by gently pipetting up and down, centrifuging at 500 × *g* for 3 min, and discarding the supernatant. The cell pellet was lysed using 500 μl of lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4) containing 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. The cell suspension was sonicated using five 1-s bursts at low power and incubated on ice for 30 min, vortexing every 5 min for 5 s. Cell lysates were then centrifuged at 10,000 × *g* for 2 min at 4 °C, and the clarified supernatant was transferred to a new tube. The lysate was incubated with 500 μl of a slurry of avidin beads in a column (prewashed three times in wash buffer) for 60 min at room temperature on a rocking platform. The column was centrifuged for 1 min at 1, 000 × *g*, and the flow-through was collected. Then the beads were washed three times using 500 μl of wash buffer (lysis solution) with protease inhibitors. After each wash, columns were centrifuged for 1 min at 1,000 × *g*, and the rinse was discarded. Finally to elute the biotinylated proteins from the beads, 400 μl of SDS-PAGE sample buffer (Invitrogen, LC2676) with 50 mM DTT was added to the column, it was heated in a heat block for 5 min at 95 °C, and samples were collected following centrifugation for 2 min at 1,000 × *g*.

DIGE/Biotin Targeting of Surface Proteins—DIGE/biotin labeling was carried out while cells were still anchored to the culture dishes. Briefly dishes (150 mm) were washed twice with ice-cold Hanks' balanced salt solution (HBSS; Invitrogen), pH 8.5. Reconstituted Cy5 (1 nmol/μl) was diluted in HBSS, pH 8.5, containing 1 M urea to make 1 nmol/ml concentrations. 2 ml of CyDye (in HBSS/urea solution) was added per dish (2×10^7 cells/dish, approximately 1×10^7cells/nmol of CyDye) and incubated for 15 min at 4 °C on a rocking platform. Upon completion of labeling, dishes were quickly washed three times in PBS^{2+}. Cells were then biotinylated using the method and reagents described earlier with the exception that 1 ml of L-lysine (10 mM) was added to the quenching solution after biotin labeling to quench any remaining free CyDyes.

Protein Separation—Protein samples recovered following biotin and DIGE/biotin labeling were separated by SDS-PAGE. Gels were cast with an acrylamide gradient (4–12%) using the 2DEoptimizer with a standard Laemmli Tris/glycine reagent pack (NextGen Sciences, Huntingdon, UK). Large format low fluorescence plates (1 mm, 27 × 21 cm, GE Healthcare) were used to allow subsequent visualization of fluorescent tagged proteins. After casting, the gels were overlaid with water-saturated 2-butanol and left overnight to polymerize. Next a stacking gel containing 4–5% acrylamide weakly buffered at pH 9.0 was cast over the already set resolving gel. The protein concentration of samples was determined using the Bradford assay (Bio-Rad), and equal amounts of samples (100 μg) were loaded. A constant 50-mA current was applied as proteins migrated down the stacking gel; at the stacking gel/separating gel boundary, the current was increased and maintained at 75 mA until the dye front reached the gel bottom. The total run time was ~7 h.

Protein Visualization—Gels were fixed overnight in methanol:acetic acid:water solution (4:1:5) and stained on the following day using Flamingo fluorescent stain (Bio-Rad). Briefly CyDye-tagged and Flamingo-stained protein profiles were scanned on a Typhoon 9400 imager (GE Healthcare). Cy5-tagged proteins were visualized using the red (633 nm) laser and the appropriate emission filter, 670 nm bandpass 30. Flamingo-stained proteins were scanned using the blue laser (473 nm) with 530-nm emission filter. After scanning, proteins were visualized by silver staining using the Plus one silver staining kit (GE Healthcare) with slight modifications to ensure compatibility with subsequent MS analysis (10). Finally silver-stained gels were scanned in transmission scan mode using a calibrated scanner (GS-800, Bio-Rad).

Mass Spectrometry—Gel bands were excised from silver-stained gels. In-gel digestion was performed using trypsin according to published methods for use with an Investigator ProGest (Genomic Solutions) robotic digestion system (11). Following enzymatic degradation, peptides were separated by capillary liquid chromatography on a reverse-phase column (BioBasic-18; 100 × 0.18 mm; particle size,

[1] The abbreviations used are: SMC, smooth muscle cell; esSMC, embryonic stem cell-derived smooth muscle cell; TMHMM, transmembrane hidden Markov model; TMH, transmembrane helix; Sm22, smooth muscle protein 22; NHS, *N*-hydroxysulfosuccinimide; HBSS, Hanks' balanced salt solution; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

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5 μm; Thermo Electron Corp.) at 2 μl/min using a Surveyor MS pump (Thermo Electron Corp.) and eluted with a 90-min gradient (0.1–30% B in 35 min, 30–50% B in 10 min, and 50–80% B in 5 min where A = 99.9% H_2O, 0.1% formic acid and B = 99.9% acetonitrile, 0.1% formic acid). The column was coupled to an electrospray source, and spectra were collected from an ion trap mass analyzer (LCQ Deca XP Plus, Thermo Electron Corp.) using full ion scan mode over the *m/z* range 300–1,800. MS/MS was performed on the top three ions in each MS scan using the data-dependent acquisition mode with dynamic exclusion enabled. MS/MS spectra were matched to database entries (UniProt Knowledgebase Release 7.5 consisting of UniProtKB/Swiss-Prot Release 49.5 and UniProtKB/TrEMBL Release 32.5 of April 18, 2006) using TurboSEQUEST software (Bioworks 3.3, Thermo Finnigan). All peptide sequence assignments were required to result from fully tryptic cleavages of the corresponding proteins. Assignments were accepted when the Xcorr score was >1.5 for singly charged ions, >2.0 for doubly charged ions, and >2.5 for triply charged ions along with ΔCn values ≥0.1, peptide probability $<1 \times 10^{-3}$, and number of distinct peptides ≥2.

Confocal Microscopy—Smooth muscle cells were cultured in 8-well chamber slides, seeding each well with 10,000 cells. Once cells adhered they were CyDye-labeled. Briefly cells were washed twice with ice-cold HBSS, pH 8.5, and labeled using CyDye in HBSS/urea solution (1 pmol/10,000 cells). Following an incubation time of 15 min at 4 °C on a rocking platform, the reaction was quenched using L-lysine (10 mM). Slides were quickly rinsed in PBS, and membrane integrity was assessed by incubating cells for an additional 5 min with propidium iodide (10 μg/ml) in a solution containing 10 mM Hepes, 100 mM NaCl, 2 mM $CaCl_2$ (pH 7.4). Finally cells were rinsed in PBS, mounted, visualized, and imaged using a Leica TCS SP5 confocal microscope.

Western Blotting—To assess the purity of the enriched membrane fractions equal protein concentrations (Bradford assay, Bio-Rad) of avidin flow-through fractions (intracellular proteins) and of resin-bound fractions (membrane proteins, 25 μg) were separated on 4–12% Tris/glycine precast gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked (overnight at 4 °C) in 5% milk in PBS. Consequently they were probed with the appropriate primary antibodies (1 h at room temperature) diluted in 5% milk in PBS, washed three times for 5 min each in 0.05% Tween 20 in PBS, and incubated again (1 h at room temperature) with appropriate horseradish peroxidase-conjugated secondary antibodies in 5% milk in PBS. Membranes were washed three times for 5 min each, ECL (GE Healthcare) was applied for 1 min, and proteins were detected using x-ray films.

Immunocytochemical Staining—SMCs and esSMCs were cultured in 8-well chamber slides (10,000 cells/well). After washing twice with cold PBS, the cells were fixed with cold 4% paraformaldehyde in PBS for 10 min at room temperature. Next 3% H_2O_2 in methanol was used to quench endogenous peroxidase for 30 min. Cells were then washed in PBS two times for 5 min each and then incubated with normal swine serum (5% in PBS) for 20 min at room temperature. The excess serum was drained, and cells were incubated for 1 h with anti-E-cadherin monoclonal antibody (Abcam) in a 1:500 dilution. The cells were washed twice in PBS for 5 min each. The cells were incubated for 30 min with swine anti-rabbit Ig-horseradish peroxidase in a 1:100 dilution. Next the cells were washed twice in PBS for 5 min each and incubated in peroxidase substrate solution (3,3′-diaminobenzidine, Dako, K3466) for 2 min. The cells were rinsed in water and mounted using DPX mounting medium (Fluka, 44581).

RT-PCR—Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed using an Improm-II™ RT kit (Promega, Madison, WI). cDNA (50 ng) was used in a PCR kit (Invitrogen) following the manufacturer's instructions. Oligonucleotide primer sequences were as follows: aortic smooth muscle actin (*Acta2*): forward, 5′-ACGGCCGCCTCCTCTTCCTC-3′; reverse, 5′-GCCCAGCTTCGTCGTATTCC-3′; smooth muscle protein 22 (*Sm22*): forward, 5′-GCAGTCCAAAATTGAGAAGA-3′; reverse, 5′-CTGTTGCTGCCCATTTGAAG-3′; calponin: forward, 5′-ACCAACCATACACAAGTTCAGTCC-3′; reverse, 5′-CCAATGATGTTCCTGCCTTCTCTC-3′; *Nanog*: forward, 5′-AGGGTCTGCTACTGAGATGCTCTG-3′; reverse, 5′-CAACCACTGGTTTTTCTGCCACCG-3′; *Rex1*: forward, 5′-GGCCAGTCCAGAATACCAGA-3′; reverse, 5′-GAACTCGCTTCCAGAACCTG-3′; *Oct4*: forward, 5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′; reverse, 5′-CTCGAACCACATCCTTCTCT-3′; *Gapdh*: forward, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′; reverse, 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′; β-actin (*Actb*): forward, 5′-CACAACTGGGACGACATGGAG-3′; reverse, 5′-TTCATGAGGTAGTCAGTCTGG-3′.

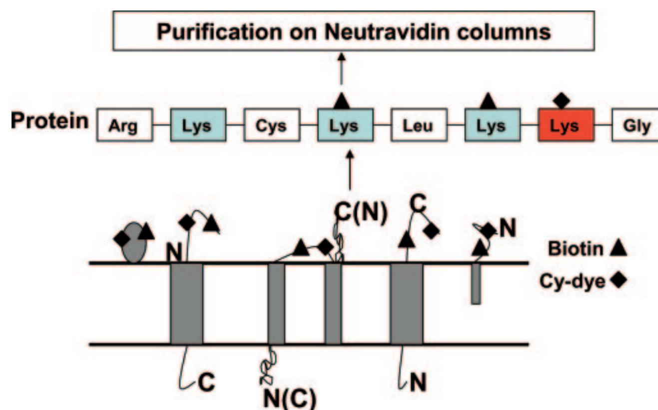

FIG. 1. **DIGE/biotin labeling.** Plasma membrane proteins are targeted with a combination of DIGE/biotin labeling. After labeling of lysines using CyDye DIGE fluor minimal dyes, remaining lysines are targeted using biotin and consequently purified on avidin affinity columns.

RESULTS

The DIGE/Biotin Labeling Procedure—We attempted to combine DIGE and biotin labeling of membrane proteins based on the principle that both techniques use an *N*-hydroxysulfosuccinimide (NHS) ester group to react with the ε-amine of lysines. Because CyDye DIGE fluor minimal dyes are designed to ensure the dyes are limiting in the reaction (12), biotin can target the remaining free lysines, although the stoichiometry has to be kept low during primary amine derivatization so as not to compromise trypsinolysis. Subsequent purification of biotinylated proteins by avidin affinity columns allows enrichment of fluorescent tagged membrane proteins. Fig. 1 shows an overview of the combined DIGE/biotin labeling approach.

Enrichment of Plasma Membrane Proteins—Plasma membrane proteins of SMCs were targeted using DIGE/biotin and biotin labeling. Following avidin purification the eluate (membrane) and flow-through (intracellular) fractions were collected. Both fractions were analyzed by immunoblotting using antibodies to membrane (integrin β1, neural cell adhesion molecule, and transforming growth factor β receptor II) as well as intracellular proteins (α-tubulin, lactate dehydrogenase, and heat shock protein 90). As shown in Fig. 2, only trace

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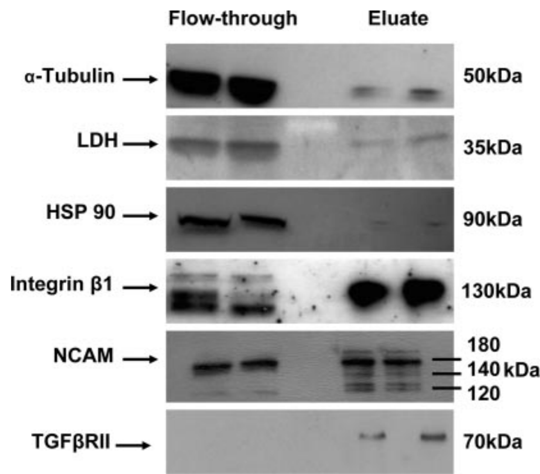

FIG. 2. **Enrichment of membrane proteins using DIGE/biotin and biotin labeling approaches.** Differences in protein expression for intracellular and membrane proteins (eluates and flow-throughs) following avidin purification were assessed by immunoblotting. For biotin and DIGE/biotin samples, the membrane proteins integrin β1, neural cell adhesion molecule (*NCAM*), and transforming growth factor β receptor II (*TGFβRII*) were highly expressed in the elution fractions. In contrast, the intracellular proteins α-tubulin, lactate dehydrogenase (*LDH*), and heat shock protein 90 (*HSP 90*) were highly expressed in the flow-through fraction. For membrane proteins, the isoforms with the largest cytoplasmic domains were identified in the flow-through: for neural cell adhesion molecule, this was the 180-kDa isoform, whereas for integrin β1, these were the 100- and 78-kDa isoforms. The data shown are representative of three similar experiments.

amounts of membrane proteins were identified in the flow-through fractions, whereas intracellular proteins were substantially reduced but not completely removed in the membrane protein fraction. Notably control experiments with unbiotinylated extracts revealed that enrichment was dependent on biotinylation and not a result of unspecific binding of membrane proteins to avidin affinity columns (Supplement 1).

Protein Separation and Visualization—Following SDS-PAGE separation of eluates, the gels were scanned on a Typhoon imager using the 633 nm laser with narrow bandpass filters to resolve Cy5-tagged proteins (Fig. 3*A*, *red*) in the DIGE/biotin samples. The 532 nm laser was used to visualize the counterstaining for total proteins (*green*, Flamingo stain, Bio-Rad). Samples labeled with biotin only served as control (Fig. 3*A*, *left lane*) to demonstrate that the enrichment was not compromised by the fluorescent labeling. Although the total protein stain of the biotin and DIGE/biotin samples resembled each other, the DIGE/biotin sample highlighted specific bands amid the biotinylated proteins that were additionally CyDye-tagged (*red*). Importantly reproducible fluorescence profiles were obtained when using constant CyDye concentrations (Supplement 2). Fig. 3*B* shows the separate fluorescence channels obtained for Cy5-tagged and total proteins (Flamingo) in the DIGE/biotin sample along with a corresponding silver-stained image. As the band pattern in Flamingo and

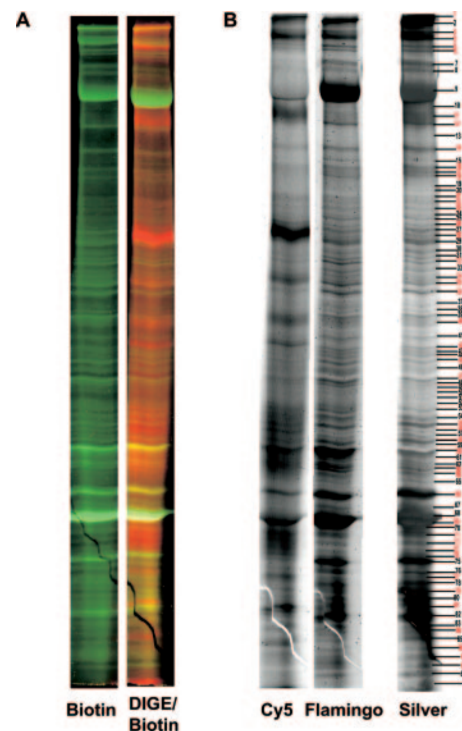

FIG. 3. **Visualization of proteins following SDS-PAGE separation.** Following SDS-PAGE and Flamingo staining of biotin and DIGE/biotin samples, gels were scanned on a Typhoon scanner. *A*, side-by-side comparison of fluorescent protein profiles of biotin samples (Flamingo-stained) compared with an overlaid fluorescent scan (CyDye and Flamingo) of DIGE/biotin samples. Note that the general protein patterns of the biotin (*green*, Flamingo stain) and DIGE/biotin samples resemble each other with the exception that in the DIGE/biotin sample certain proteins are CyDye-tagged (*red*). *B*, the separate scanning channels for detection of CyDye- and Flamingo-tagged proteins within DIGE/biotin samples along with a silver-stained protein profile. As the band pattern in Flamingo and silver staining was identical, Cy5-tagged bands could be accurately aligned, and silver-stained bands corresponding to CyDye-tagged and non-tagged proteins were easily discerned and numbered in *red* and *black*, respectively. The data shown are representative of two similar experiments.

silver staining was identical, the Cy5-tagged bands could be accurately aligned. This allowed silver-stained bands corresponding to CyDye-tagged and non-tagged proteins to be easily discerned, and these are numbered in *red* and *black*, respectively (Fig. 3*B*).

Protein Identification—The bands numbered in Fig. 3*B* were excised and subjected to in-gel tryptic digestion using a robotic digester (ProGest, Genomic Solutions). Each digest was analyzed by LC-MS/MS using a 90-min gradient. MS/MS spectra were searched against the UniProt database using the Sequest algorithm. From the 90 bands, a total of 228 proteins were identified by multiple peptides (Supplement 3); those corresponding to the CyDye labeled bands (Fig. 3*B*, *red numbers*) are shown separately in Supplement 4. A comparison in terms of protein functionality and localization between CyDye-tagged bands (*red numbers*) and non-tagged bands

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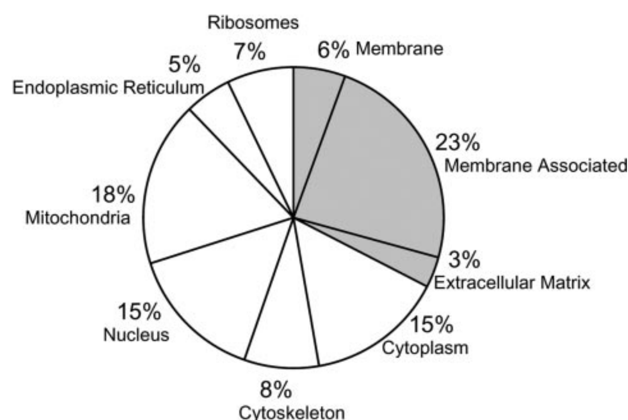

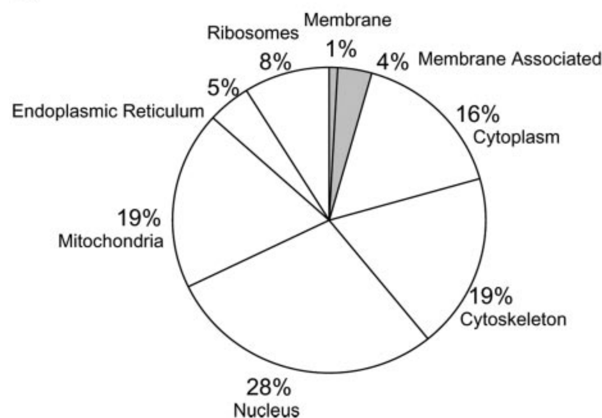

FIG. 4. **Comparison of identified CyDye-tagged and non-tagged proteins.** The subcellular localization of identified CyDye-tagged fluorescent proteins (*A*) and non-tagged proteins (*B*) was determined based on the Swiss-Prot database. The percentage of the proteins in each category is identified. In CyDye-tagged fluorescent bands (*A*), we achieved an overall increase from 5 to 32% within the groups of membrane, membrane-associated, and extracellular matrix proteins and a corresponding reduction in intracellular proteins (cytoskeleton, cytoplasm, mitochondria, nucleus, endoplasmic reticulum, and ribosomes).

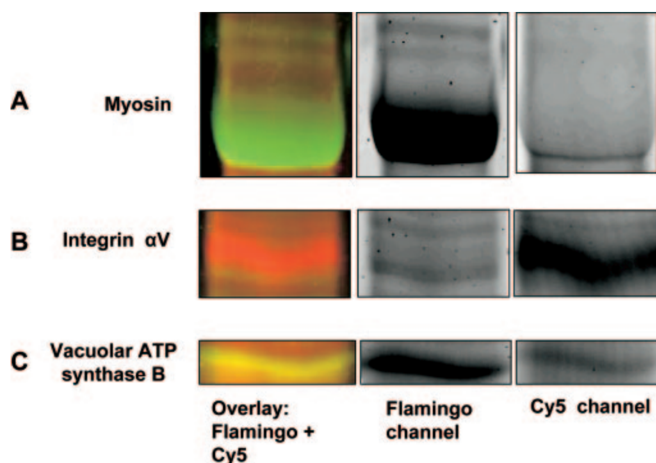

FIG. 5. **Enlarged image of the DIGE/biotin sample.** *A*, abundant intracellular proteins such as myosin (band 9, see Fig. 3*B*) were not CyDye-tagged. *B*, in contrast, membrane proteins like integrin αV (band 28, see Fig. 3*B*) are barely visible with Flamingo total protein stain but intensely CyDye-labeled. *C*, LC-MS/MS analysis of band 60 (see Fig. 3*B*), which was labeled by both Flamingo stain and CyDye tagging, confirmed the presence of the membrane protein vacuolar ATP synthase B among intracellular proteins (see Supplement 3).

(*black numbers*) is shown in Fig. 4, *A* and *B*, respectively. Regarding the groups of membrane, membrane-associated proteins, and extracellular matrix proteins, we achieved an overall increase from 5% in non-tagged bands to 32% in CyDye-tagged bands and a corresponding reduction in intracellular proteins (predominantly cytoskeleton and nucleus). The specificity of the DIGE/biotin labeling toward these three classes of proteins is further highlighted in Fig. 5. A band identified as myosin was labeled by Flamingo staining (*green*) but not by CyDye (Fig. 5*A*). In contrast, the cell adhesion molecule integrin αV has low intensity in the total protein stain but is strongly CyDye labeled (Fig. 5*B*). Furthermore bands visualized by faint CyDye but prominent Flamingo staining highlighted the presence of membrane proteins among intracellular proteins (Fig. 5*C*). Notably although multiple identifications were obtained for most bands, an inherent issue of protein co-migration associated with SDS-PAGE, membrane, membrane-associated, and extracellular matrix proteins rarely co-localized within the same CyDye-tagged band (see Supplement 4), a prerequisite for semiquantitative comparisons based on the staining intensity.

Characterization of Membrane Receptors—Among the CyDye-tagged proteins were peripheral membrane proteins and transmembrane receptors including neural cell adhesion molecule 1, integrin α5, neural cadherin, integrin αV, vascular cell adhesion molecule (13, 14), vacuolar ATP synthase subunit B, and a new receptor, KIAA0152, with an unknown function. The MS-MS spectra for the known SMC membrane receptors are provided in Supplement 5. The MS-MS spectrum for the newly identified membrane protein KIAA0152 is shown in Fig. 6. For the membrane receptors, TMHMM version 2.0 (15) was used to calculate the length, number of TMHs, expected numbers of amino acids in the TMHs, and the probability that the N terminus is inside. In addition GRAVY scores (16) for these proteins were calculated. The results for these different parameters are shown in Table I. Membrane proteins targeted possessed one or two transmembrane helices, suggesting that the majority of amino acid sequences for these proteins are located on the extracellular and intracellular hydrophilic domains. Indeed GRAVY scores indicate that these proteins are hydrophilic. Given the hydropathy index of lysine (−3.9), this implies the presence of several lysine residues. Further-

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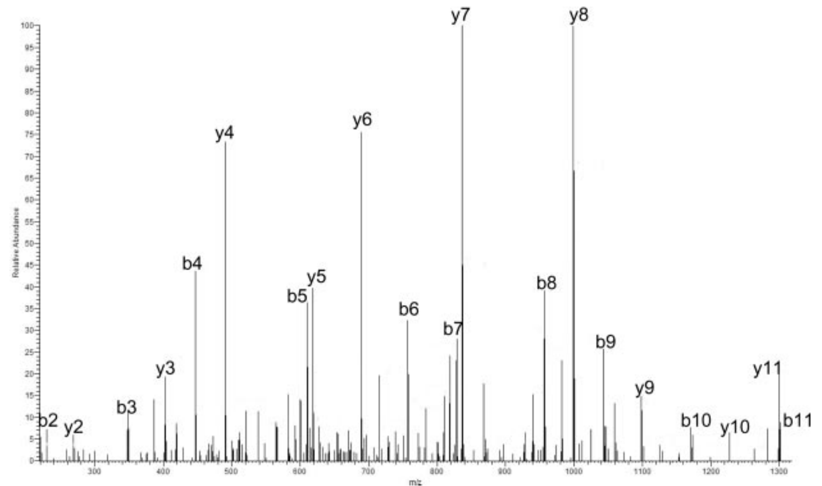

Fig. 6. **MS/MS spectrum of the KIAA0152 peptide (amino acids 126–138).** A search against the complete UniProt database returned the result that this identified peptide is specific for the mouse KIAA0152 protein. The spectrum shows the b- and y-ion series of the peptide FAEVYFAQSQQK.

Table I

Membrane protein topology (TMHMM) and GRAVY scores

Transmembrane proteins	Length	No. of predicted TMHs	Expected no. AAs[a] in TMHs	Total probability N-in[b]	GRAVY score
Neural cell adhesion molecule 1	1,115	2	44.80	0.48	−0.41
Neural cadherin precursor	906	1	23.13	0.01	−0.35
Epithelial cadherin precursor[c]	889	1	23.08	0.001	−0.42
Integrin α5 precursor	1,053	1	22.91	0.003	−0.23
Integrin αV precursor	1,044	1	23.11	0.01	−0.25
Integrin α6[c]	1,091	1	25.12	0.09	−0.39
Vascular cell adhesion protein 1	739	1	36.69	0.74	−0.10
Protein KIAA0152	291	1	24.91	0.29	−0.20
4F2 (CD98)[c]	526	1	22.52	0.98	−0.21

[a] Amino acids.
[b] The total probability that the N terminus is on the cytoplasmic side of the membrane.
[c] Identified in esSMCs.

more the probability scores suggested that in many of the membrane proteins identified the N termini face the extracellular environment, an observation consistent with previous reports stating that type 1 membrane proteins are preferentially targeted by biotin labeling (17).

Characterization of Membrane-associated Proteins—Apart from membrane receptors, extracellular matrix proteins, *i.e.* collagen and fibronectin (14), and membrane-associated proteins were also identified. The majority of proteins belonging to the latter category were cell adhesion molecules known to bind tightly to their receptors, cadherins, and integrins. Based on the identified proteins we were able to reconstruct several known pathways involved in vascular SMC adhesion (Fig. 7). When the DIGE surface labeling was subsequently assessed using confocal microscopy (Fig. 8), the staining pattern displayed a dotted, granular, and interrupted staining pattern similar to what has been previously described for various cell adhesion (18, 19) molecules.

Membrane Protein Differential Expression Analysis—To establish the applicability of the DIGE/biotin approach for differential expression analysis of membrane proteins, a comparison of esSMCs and mature SMCs was performed. Both cell lines were DIGE/biotin-labeled using Cy5, and samples were separated by SDS-PAGE. Fig. 9*A* shows the CyDye-tagged protein profiles of SMCs and esSMCs, respectively. The CyDye-tagged bands specific to esSMCs were numbered, excised, and analyzed by mass spectrometry (Fig. 9*A*). Protein identifications are provided in Table II. We successfully identified three membrane receptors (CD98, integrin α6, and E-cadherin) of the five bands excised. Again it can be observed that not more than one protein from the membrane, membrane-associated, and extracellular categories was localized within the same band and that the membrane protein topology of these proteins was similar to the topology of the proteins identified in SMCs (see Table I). To confirm whether the three membrane receptors were indeed predominantly

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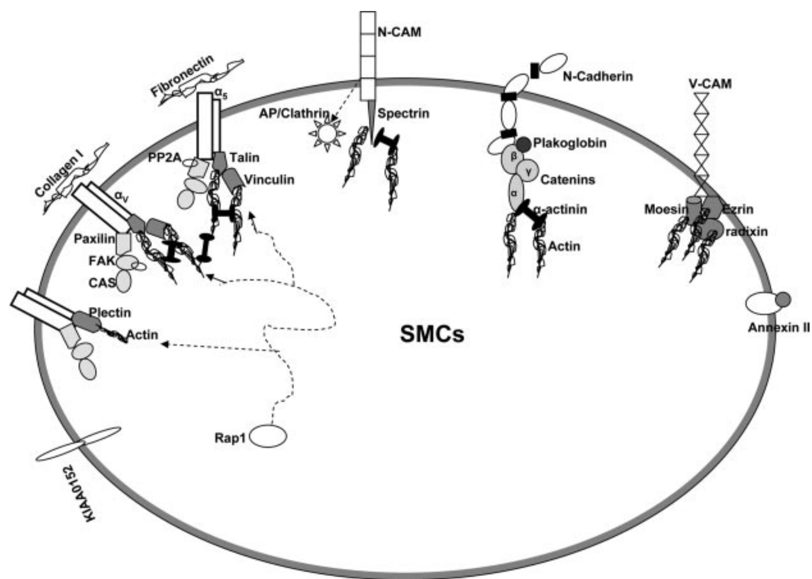

FIG. 7. **Identified membrane and membrane-associated proteins.** The receptors and their associated intracellular and extracellular interacting proteins identified in the present study were used to reconstruct known pathways in SMCs. *FAK*, focal adhesion kinase; *CAS*, Crk-associated substrate; *PP2A*, protein phosphatase 2A; *AP*, assembly protein; *N-CAM*, neural cell adhesion molecule; *V-CAM*, vascular cell adhesion molecule.

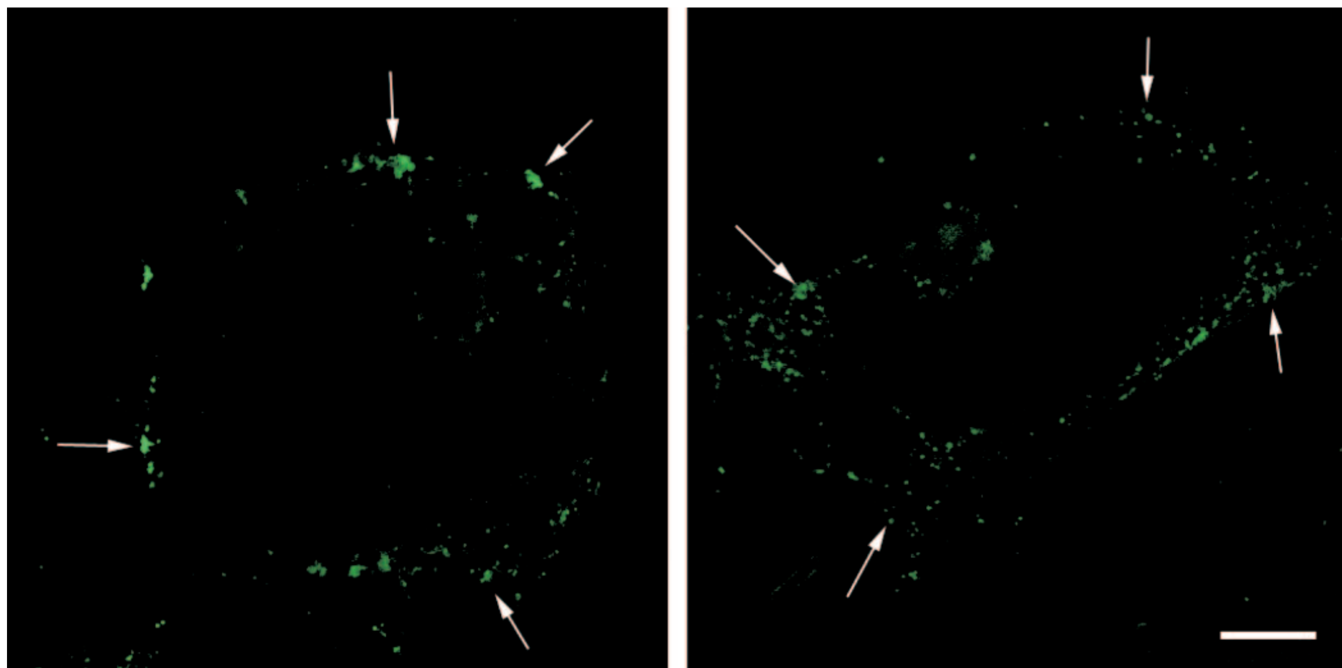

FIG. 8. **Confocal microscopy.** SMCs were surface-labeled with Cy2 (*green*) and consequently incubated with propidium iodide to assess the cell membrane integrity. Cells were imaged using a Leica TCS SP5 confocal microscope. The *white arrows* point to a dotted, granular, and interrupted staining pattern restricted to the plasma membrane. The data shown are representative of three similar experiments. *Bar*, 10 μm.

expressed in esSMCs, Western blot analysis and immunocytochemical staining were performed. Immunoblots (Fig. 9*B*) of eluates showed that 4F2 (CD98) was more abundant in esSMCs. Similarly for integrin α6, the proform (140 kDa) was predominantly expressed in esSMCs. With regard to E-cadherin, immunocytochemical staining (Fig. 9*C*) confirmed higher expression of this cell adhesion protein in esSMCs than in mature SMCs. Overall the identified membrane proteins indicate that esSMCs differ from mature SMCs, and this was further supported by RT-PCR results. EsSMCs expressed SMC markers (calponin, *Sm22*, and aortic smooth muscle actin) (Fig. 9*D*) but also showed persistent expression of embryonic stem cell pluripotency markers (*Oct4*, *Nanog*, and *Rex1*).

DISCUSSION

In this study, we propose a new method of profiling membrane proteins based on two existing technologies for membrane protein purification and labeling, both of which are readily available in most laboratories but in combination sim-

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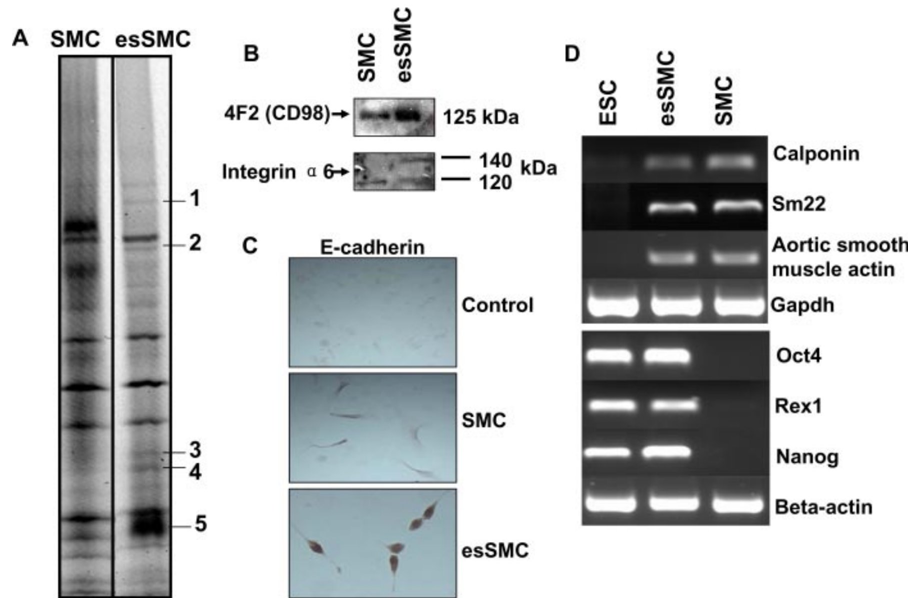

FIG. 9. **DIGE/biotin membrane differential expression analysis.** *A*, CyDye-tagged protein profiles of SMCs and esSMCs, respectively. CyDye-tagged proteins, which appeared to be expressed exclusively by esSMCs, were numbered, excised, and analyzed by mass spectrometry. *B*, Western blots on eluates of SMCs and esSMCs showed that 4F2 (CD98), consisting of the light and heavy chains, had a higher expression in esSMCs. For integrin $\alpha 6$, the proform (uncleaved) isoform (140 kDa) was only expressed in esSMCs, whereas the heavy chain (120 kDa) was present in both cell lines. *C*, immunocytochemical staining confirmed the increased abundance of E-cadherin in esSMCs compared with aortic SMCs. *D*, SMC markers (calponin, *Sm22*, and aortic smooth muscle actin) and pluripotency markers (*Oct4*, *Nanog*, and *Rex1*) were assessed by RT-PCR on undifferentiated embryonic stem cells (*ESC*), esSMCs, and SMCs. *Gapdh* and β-actin served as a loading control.

TABLE II
Protein identifications for differentially expressed DIGE/biotin bands in esSMCs

No.	Protein identity	Swiss-Prot entry name	Peptides identified[a]	Molecular mass	XC[b]	Subcellular location
				Da ($\times 10^3$)		
1	Integrin α 6 precursor	ITA6_MOUSE	K.NIGDINQDGYPDIAVGAPYDDLGK.V	122.0	2.65	Membrane receptor
			R.VNSLPEVLPILNSNEAK.T		2.96	
2	U5 small nuclear ribonucleoprotein component	U5S1_MOUSE	K.STPVTVVLPDTK.G	109.2	2.29	Nucleus
			K.IAVEPVNPSELPK.M		3.31	
	Epithelial cadherin precursor	CADH1_MOUSE	K.DINDNAPVFNPSTYQGQVPENEVNAR.I	98.1	3.72	Membrane receptor
			R.RVEVPEDFGVGQEITSYTAR.E		4.27	
			R.PANPDEIGNFIDENLK.A		4.76	
			R.DTGVISVLTSGLDR.E		4.52	
3	Ezrin	EZRI_MOUSE	R.QLLTLSNELSQAR.D	69.3	4.10	Cytoskeleton/membrane-associated
			K.APDFVFYAPR.L		3.76	
4	4F2 cell surface antigen heavy chain	4F2_MOUSE	R.IGDLQAFVGR.D	58.3	3.06	Membrane receptor
			R.LGASNLPAGISLPASAK.L		3.05	
5	Guanine nucleotide-binding protein G_i, α-2 subunit	GNAI2_MOUSE	R.IAQSDYIPTQQDVLR.T	40.4	3.78	Membrane-associated
			K.LLLLGAGESGK.S		3.64	
	Calponin-3	CNN3_MOUSE	K.AGQSVIGLQM*GTNK.C	36.4	3.06	Cytoskeleton
			K.LTLQPVDNSTISLQM*GTNK.V		4.45	

[a] M* denotes oxidation of methionine.
[b] X correlation score.

plify gel-based analysis of membrane proteins. Our combined approach using CyDye labeling and subsequent membrane protein enrichment based on biotin/avidin purification offers several advantages. First, in this new approach, analysis can be confined to those proteins that are fluorescently labeled. As observed in the biotin approach alone, fluorescent labeling is particularly important because membrane proteins, due to their low abundance, are often present in bands of very low silver staining intensity even after enrichment. With our combined approach, the fluorescence tag will pinpoint the bands containing membrane proteins. Second, we have shown that this approach can be used for semiquantitative differential

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expression of membrane proteins. A side-to-side comparison of the CyDye-tagged protein profiles readily identified bands specific to esSMCs, which were subsequently shown to contain membrane receptors with increased abundance in the stem cell-derived cells. Notably without additional CyDye tagging these bands would have been impossible to depict from samples labeled with just biotin because contaminating cytoskeletal components still dominate the total protein staining pattern.

Previously our group created a dataset of proteins expressed in vascular aortic SMCs (20), Sca-1$^+$ progenitors derived from embryonic stem cells (21), and conducted a DIGE analysis between esSMCs and SMCs using two-dimensional electrophoresis (9). Notably none of the membrane, membrane-associated, and extracellular proteins identified in the present study was among these datasets. This indicates that the DIGE/biotin labeling targets a different subcellular proteome. With regard to the profiling of SMCs, the majority of membrane, extracellular, and membrane-associated proteins identified were involved in cell adhesion. This finding is further supported by confocal images of surface-labeled SMCs revealing a staining pattern characteristic of cell adhesion molecules. We also identified a single pass type I membrane protein, KIAA0152, with an unknown function. The protein has been shown to be very close in homology to KOG3593, a receptor-like serine/threonine kinase domain, suggesting a role for this protein in signal transduction mechanisms (22). Another member of the KIAA family, KIAA0747, with a similar potential function was recently classified as a new receptor on platelets (23). Interestingly a bioinformatics analysis of uncharacterized genes in heart failure revealed a 12.5-fold up-regulation of KIAA0152 in disease, and Center for Biological Sequence Analysis SignalP Prediction Server search results indicated that KIAA0152 possessed six serine, three threonine, seven tyrosine phosphorylation sites and one glycosylation site (22). However, the function of this protein in heart failure and vascular SMCs remains to be elucidated.

Finally using the DIGE/biotin platform, membrane differential expression analysis between esSMCs and SMCs was performed. The rationale for conducting this specific comparison stands from findings of a previous study by our group that revealed that the cellular proteome of the two cell types was significantly different, although esSMCs expressed a panel of smooth muscle markers (9). Results from this investigation suggested the need for new markers not only capable to assess the stage of the stem cell differentiation process but to clearly distinguish between the mature and stem cell-derived SMCs. Using our current approach, we identified three membrane receptors (4F2, integrin α6, and E-cadherin), shown in Fig. 9, that may be used for this purpose. Notably in a proteomics study by Nunomura *et al.* (24), 4F2 (CD98) was identified among several CD antigens and membrane receptors shown to be expressed on the surface of undifferentiated mouse embryonic stem cells. In another study, Azzarone *et al.* (25) showed that 4F2 was up-regulated in human fibroblasts of embryonic origin and down-regulated as they differentiated and/or matured into adult cells. With regard to integrin α6, a study analyzing microRNA of differentiated and undifferentiated embryonic stem cells revealed that this integrin was highly expressed in undifferentiated cells and showed a quantitative shift among the splicing isoforms during the differentiation process (26). In addition, similar to our findings, a study on differentiating embryonic lens fiber cells has shown that the uncleaved proform was present on the cell surface and that as the cells differentiated there was a switch in predominance from the cleaved to uncleaved protein (27). Finally E-cadherin has been found to be strongly expressed homogenously in undifferentiated mouse embryonic stem cells (28) with a primary function of mediating attachment between neighboring embryoid bodies; however, during differentiation, the receptor was found to be down-regulated (29). Relating these findings to our own, which show an overexpression of CD98, uncleaved integrin α6, and E-cadherin in esSMCs, we may presume that esSMCs maintain characteristics of their embryonic stem cell origin despite expressing the same SMC markers as mature aortic SMCs. This hypothesis was further confirmed by our finding that esSMCs retained the expression of pluripotency markers (*Oct4*, *Nanog*, and *Rex1*), an important feature of embryonic stem cells.

In summary, this study was intended to develop a simple method to study membrane proteins by utilizing fluorescence labeling together with the purification capabilities of the biotin/avidin extraction for integrated membrane protein analysis. We achieved efficient labeling of membrane proteins and their associated proteins and provide proof-of-principle for the suitability of the platform for semiquantitative differential expression analysis by identifying membrane proteins that can serve as additional markers during mouse embryonic stem cell differentiation. We expect membrane proteomics to offer an opportunity to progress toward a more comprehensive classification of stem cell-derived cells (30, 31).

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