Serumproteomik zur Identifizierung tumorspezifischer Biomarker des nicht-kleinzelligen Bronchialkarzinoms

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

DOKTOR DER NATURWISSENSCHAFTEN

Dr. rer. nat.

genehmigte Dissertation von

Dipl.-Biol. Bijon Gopal Chatterji

geboren am 30.12.1978 in Bremen

2009

Referent: Prof. Dr. Jürgen Borlak

Lehrstuhl für Pharmako- und Toxikogenomik
Medizinische Hochschule Hannover

Abteilung Molekulare Medizin und Medizinische Biotechnologie
Fraunhofer Institut für Toxikologie und Experimentelle
Medizin (ITEM), Hannover

Korreferent: Prof. Dr. Markus Kalesse

Institut für Organische Chemie
Gottfried Wilhelm Leibniz Universität Hannover

Abteilung Medizinische Chemie
Helmholtz Zentrum für Infektionsforschung (HZI), Braunschweig

Tag der Promotion: 1. Juli 2009

Die vorliegende Arbeit wurde in der Zeit von November 2004 bis November 2008 am Fraunhofer Institut für Toxikologie und Experimentelle Medizin in Hannover unter der wissenschaftlichen Leitung von Prof. Dr. Jürgen Borlak in der Abteilung für Molekulare Medizin und Medizinische Biotechnologie angefertigt.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur mit Hilfe der angegebenen Hilfsmittel und Quellen verfasst habe. Die entnommenen Stellen aus benutzen Werken wurden wörtlich oder inhaltlich als solche gekennzeichnet. Diese Dissertation wurde bisher weder als Diplom- noch sonstige Prüfungsarbeit verwendet.

Hannover, im März 2009

Meinem Vater gewidmet

Inhaltsverzeichnis

Zusammenfassung

Lungenkrebs ist die vierthäufigste Erkrankung in der Bundesrepublik Deutschland. Jährlich sterben bundesweit mehr als 40.000 Menschen daran. Damit ist der Lungenkrebs eine der häufigsten Krebserkrankungen überhaupt. Allgemein werden Lungentumore in kleinzellige (SCLC) und nicht-kleinzellige Lungenkarzinome (NSCLC) unterteilt, die zu unterschiedlichen Prognosen für das Überleben führen. Mit Hilfe transgener Tiermodelle können molekulare Mechanismen in der Pathogenese untersucht werden um die Entwicklung verbesserter Therapien vorantreiben zu können. Daher wurden im Rahmen dieser Doktorarbeit drei Mausmodelle des Lungenkarzinoms untersucht, welche entweder die Serin-/Threoninkinase c-raf, den Transkriptionsfaktor c-myc bzw. das Mitogen epidermal growth factor (EGF) überexprimieren. Mit Hilfe eines Genkonstruktes, das regulatorische Sequenzen des surfactant protein-C (SP-C) Promotors enthält, konnten die Transgene in alveolärepithelialen Zellen überexprimiert werden. Anhand zweidimensionaler Gelelektrophorese (2-DE) und Matrixunterstützter Laserdesorptions-/ionisations-Massenspektrometrie (MALDI-TOF/TOF) wurden Blutseren transgener Tiere untersucht und Proteomkartierungen angefertigt. Neben einer allgemeinen Kartierung des Serumproteoms wurden Biomarker gefunden, die entweder für atypische adenomatöse Hyperplasien, bronchiolo-alveoläre Karzinome oder papilläre Adenokarzinome der Lunge spezifisch sind. Die Identifizierung von spezifischen Biomarkern ermöglicht eine verbesserte und frühzeitige Diagnostik sowie ein Monitoren der eingeleiteten Therapien.

Schlagwörter: Lungenkrebs / Biomarker / Serumproteomik

Abstract

Lung cancer remains the leading cause of cancer death worldwide. In 2007, more than 40,000 people died from this disease in the Federal Republic of Germany alone. In general, lung tumors are classified as small cell (SCLC) or non-small cell lung carcinomas (NSCLC). Essentially, transgenic animal models are useful for an improved understanding of the molecular mechanisms of tumourigenesis. Here we investigated three transgenic mouse models where targeted overexpression of the serine/threonine kinase c-raf, the transcription factor c-myc or the mitogen epidermal growth factor (EGF) was achieved by usage of a gene construct that contains regulatory sequences of the surfactant protein-C (SP-C) promotor. Animals thus overexpressed the transgene in alveolar-epithelial cells. We analyzed blood serum of transgenic animals and report proteome maps developed by two-dimensional gel-electrophoresis (2-DE) and matrix-assisted laser-desorption/ ionization-mass spectrometry (MALDI-TOF/TOF). We identified biomarkers that are specific for atypic adenomatous hyperplasia, bronchiolo-alveolar carcinomas or papillary adenocarcinomas of the lung. The identification of novel candidate biomarkers by serum proteomics enables improved diagnostic and therapeutic monitoring of patients.

Keywords: Lung cancer / biomarkers / serum proteomics

Einleitung

Lungenkrebs ist sowohl bei Männern als auch Frauen die vierthäufigste Erkrankung in der Bundesrepublik Deutschland. Jährlich sterben bundesweit mehr als 40.000 Menschen daran (Statistisches Bundesamt, 2007). Damit ist der Lungenkrebs eine der häufigsten Krebserkrankungen überhaupt. Rauchen ist der größte Risikofaktor (relatives Risiko >30-fach) und in mehr als 80% aller Fälle für eine Tumorerkrankung verantwortlich. Weitere Risikofaktoren für eine Lungentumorerkrankung sind unter anderem eine Exposition zu radioaktiven Stoffen und die Inhalation von Schwermetallen, Asbest und Abgasen.

Lungentumore werden anhand histologischer Befunde klassifiziert und im Allgemeinen in kleinzellige (SCLC) und nicht-kleinzellige Lungenkarzinome (NSCLC) unterteilt. Neben dem Plattenepithelkarzinom und den großzelligen Karzinomen sind auch Adenokarzinome der Lunge zu beklagen, die weiterhin in verschiedene Subtypen untergliedert werden. Primär entwickeln sich Lungentumore aus dem respiratorischen Epithel, während alveolärepitheliale Adenokarzinome des Menschen eher selten sind. Neuere Daten deuten auf einen signifikanten Anstieg von Alveolar- und Clara Zell-Tumoren, die nach Schätzungen bis zu 30% aller Adenokarzinome ausmachen. Die Überlebenschancen eines Patienten mit einem Lungenadenokarzinom liegen bei nicht einmal 10% innerhalb von 5 Jahren.

Die molekularen Mechanismen in der Entstehung von Lungentumoren sind unklar und treten sporadisch auf. Nur 12 bis 15% der tumorerkrankten Patienten können mit gegenwärtigen Therapieoptionen geheilt werden. Das Spektrum umfasst die operative Entfernung von erkranktem Gewebe sowie Strahlen- und Chemotherapie. In den meisten Fällen jedoch ist die Metastasierung des Tumors soweit im Körper fortgeschritten, dass die Heilungschancen gering sind. Zwar zeigt eine Chemotherapie nach erfolgtem operativem Eingriff in frühem Krankheitsstadium eine längere Überlebensrate, doch sind die gegenwärtigen medikamentösen und radiologischen Behandlungen nicht ausreichend, um das

Krebsgewebe nachhaltig zu zerstören und den letztendlich tödlichen Ausgang zu verhindern.

Entscheidende Ansätze für ein verbessertes Verständnis der molekularen Ursachen liefern transgene Tiermodelle. Aufgrund ihrer mehr als 99%igen genetischen Ähnlichkeit zum Menschen werden Mäuse in der biomedizinischen Forschung routinemäßig eingesetzt. Gentechnisch veränderte Mäuse ermöglichen mechanistische Untersuchungen, so beispielsweise die Relevanz überexprimierter Onkogene oder das Abschalten von Tumorsupressorgenen in der Tumorentstehung. Basierend auf genetischen Untersuchungen an Patientenmaterialien wurden zahlreiche Mausmodelle für Lungentumore entwickelt für die Suche und Validierung neuer Therapien.

Im Rahmen dieser Doktorarbeit wurden drei transgene Mausmodelle untersucht, welche entweder die Serin-/Threoninkinase c-raf, das Protoonkogen c-myc oder den epidermal growth factor (EGF) überexprimieren. Durch Verwendung eines maßgeschneiderten Genkonstruktes, das regulatorische Sequenzen des surfactant protein-C (SP-C) Promotors enthält, konnten die Transgene gezielt in alveolärepithelialen Zellen exprimiert werden.

Da jeder Zelltyp in der Lage sein muss, auf extrazelluläre Signale zu reagieren, bedienen sich Zellen definierter Signalübertragungsketten, die häufig über die Aktivierung von Rezeptoren und Phosphorylierungskaskaden zu einer Zellantwort, beispielsweise einer veränderten Genexpression führen. Zu den am besten untersuchten Signalübertragungswegen gehört die Mitogen-Aktivierte Protein Kinase (MAPK)-Signaltransduktion (Abb. 1). Dabei kommt dem Transkriptionsfaktor c-myc eine große Bedeutung bei der Regulation von Zellzyklus und Apoptose zu, jedoch sind die Proteinkinase c-raf und der EGF als Ligand der EGFR Tyrosinkinase von besonderer Relevanz, wie etwa bei der Entstehung von Lungentumoren.

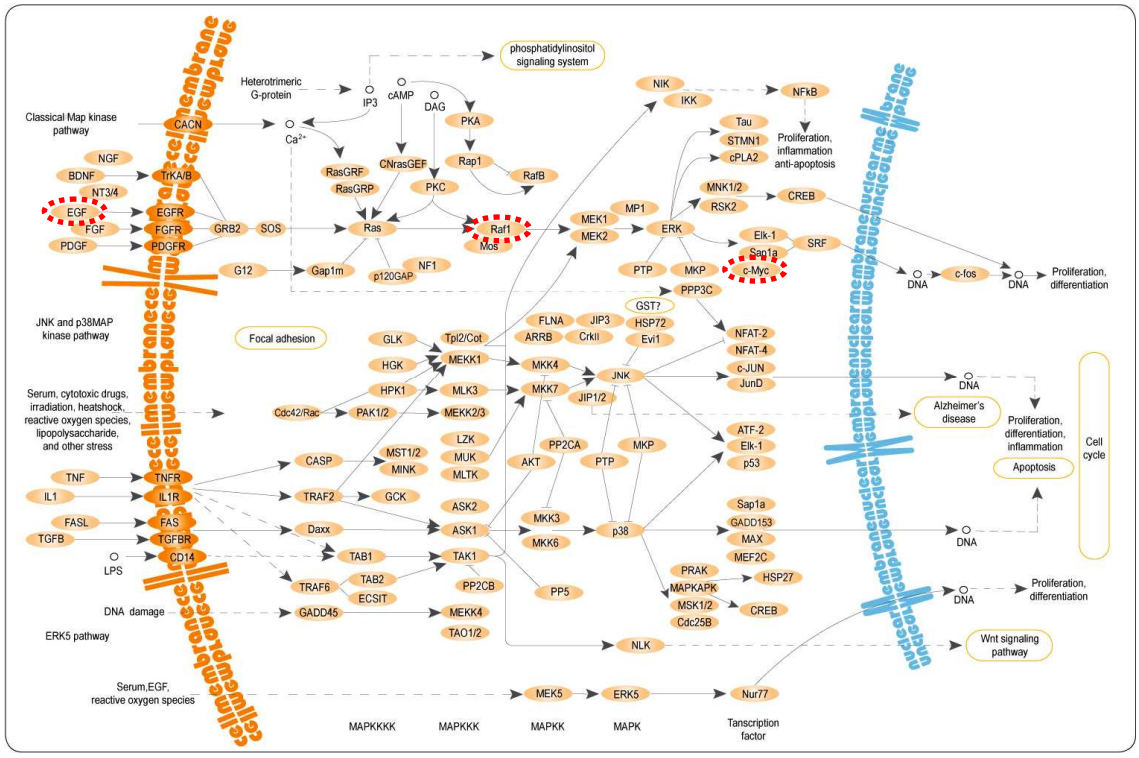


Abb. 1: MAPK Signalübertragungskette. Der Signalweg der Mitogen-Aktivierten Protein Kinasen (MAPK) spielt bei der Entstehung von Lungentumoren eine übergeordnete Rolle. MAP-Kinasen regulieren durch mitogen-vermittelte extrazelluläre Stimulation über diverse Phosphorylierungsschritte viele zelluläre Prozesse wie Genexpression, Mitose, Zelldifferenzierung, das Überleben einer Zelle oder Apoptose. Schlüsselmoleküle sind insbesondere der epidermal growth factor (EGF) als Ligand der membrangebundenen Rezeptortyrosinkinase epidermal growth factor receptor (EGFR), die Serin-/Threoninkinase c-raf (Raf1) sowie der Transkriptionsfaktor und das Protoonkogen c-myc. Quelle: Abgent Inc., San Diego, USA.

So sind in den c-raf transgenen Mäusen morphologische Veränderungen bereits früh (ca. 3-4 Monate) zu erkennen, während in weiter untersuchten Mausmodellen transgen-spezifische Tumortypen erst nach 8-10 Monaten erkennbar sind. Mittels "disease proteomics" können Proteine, die krankheitsbedingt reguliert sind, identifiziert und näher charakterisiert werden, wodurch neue Erkenntnisse in der molekularen Pathogenese der Lungentumore gewonnen werden.

Ziel der Forschungsarbeiten im Rahmen dieser Doktorarbeit war daher die Identifizierung von Proteinen, die in Lungentumoren exprimiert werden. Mit Hilfe genomweiter Genexpressionsanalysen konnten bereits zahlreiche regulierte Gene in Lungentumoren identifiziert werden. Deren Beitrag zur Krankheitsprogression wird derzeit detailliert untersucht.

In der vorliegenden Doktorarbeit wird deshalb nach diagnostischen und krankheitsrelevanten prognostischen Biomarkern gesucht. Wesentlich ist deren Quantifizierbarkeit, um biologische Prozesse vorhersagen und detektieren zu können. Insgesamt wird weltweit nach diagnostischen Biomarkern gesucht, um Tumore bereits früh erfassen und optimale Therapiekonzepte entwickeln zu können. Der Nutzen von Biomarkern für die frühzeitige Erkennung von Adenokarzinomen wurde bereits in der Literatur ausführlich diskutiert.

Die Proteomforschung ist somit nützlich, um neue Biomarker zu finden, durch beispielsweise vergleichende Untersuchungen des Serumproteoms von kranken und gesunden Tieren, mit dem Ziel tumorspezifische Proteine zu lokalisieren und sowohl deren Expression als auch Transport im Blut für diagnostische Zwecke zu nutzen. Der Einsatz der Methoden der Proteomforschung in klinischen Laboratorien ermöglicht die Identifizierung von Serummarkern für eine prognostische und diagnostische Klassifizierung von Krankheitsstadien, aber auch einen Therapieverlauf zu monitoren.

Die Tumorproteome von c-raf, c-myc und EGF induzierten Adenokarzinomen der Lunge sind unbekannt. Mittels integrierter Methoden der Proteomforschung ist es jedoch möglich speziell regulierte Proteine zu finden und zu charakterisieren.

Zielsetzung der Doktorarbeit

Folgende Ziele wurden in dieser Doktorarbeit verfolgt:

1. Proteomkartierung der Serumproteine gesunder Mäuse
2. Proteomkartierungen des Serums der transgenen Mausmodelle SP-C/c-raf, SP-C/c-myc und SP-C/IgEGF
3. Neue Erkenntnisse über die Rolle von c-raf, c-myc und EGF in der Pathogenese der Adenokarzinome der Lunge
4. Aufbau einer Serumproteomdatenbank als Referenz für weiterführende Studien mit transgenen Mausmodellen
5. Identifizierung, Validierung und Quantifizierung regulierter krankheitsassoziierter Proteine
6. Vergleich der regulierten Proteine zwischen den genannten Tiermodellen zur Identifizierung sowohl universeller als auch selektiver Biomarker zur Erkennung unterschiedlicher Stadien der Lungentumore

Eingesetzte Methoden in der Doktorarbeit

Die Blutentnahme von transgenen Tieren erfolgte über die *Vena cava*. Die Proteinkonzentration der zu untersuchenden Serumproben wurde mittels Bradford Test bestimmt. Mit einem thioharnstoffhaltigen Lysepuffer (5 mol/L urea, 2 mol/L thiourea, 40 mmol/L Tris, 4% CHAPS, 100 mmol/L DTT und 0.5% BioLyte 3-10) wurden Serumproteine von transgenen und gesunden Kontrolltieren extrahiert und mittels zweidimensionaler Gelelektrophorese (2-DE) unter Verwendung der pH-Bereiche 4-7, 5-8 und 3-10 entlang ihres individuellen isoelektrischen Punkts und nach Molekulargewicht aufgetrennt. Nach kolloidaler Coomassie-Blaufärbung der 2-D Gele wurden die gefärbten Gelspots mittels elektronischer Bild-analyseverfahren quantifiziert und statistisch signifikante Unterschiede zwischen gesunden und tumorerkrankten Mäusen festgestellt. Anschließend wurden diese Spots mit einem Spotcutter ausgeschnitten, entfärbt und tryptisch verdaut. Die dadurch gewonnenen Peptide einzelner Proteinspots wurden mit Hilfe der matrixunterstützten Laserdesorptions-/ionisations-Massenspektrometrie (MALDI-TOF/TOF) sowie LC-Massenspektrometrie (LC-ESI-MS) identifiziert (Abb. 2). Proteinexpressionen wurden mittels Western Immunoblotting validiert. Des weiteren wurden *in silico* Untersuchungen von Promoterregionen mittels Bioinformatik durchgeführt.

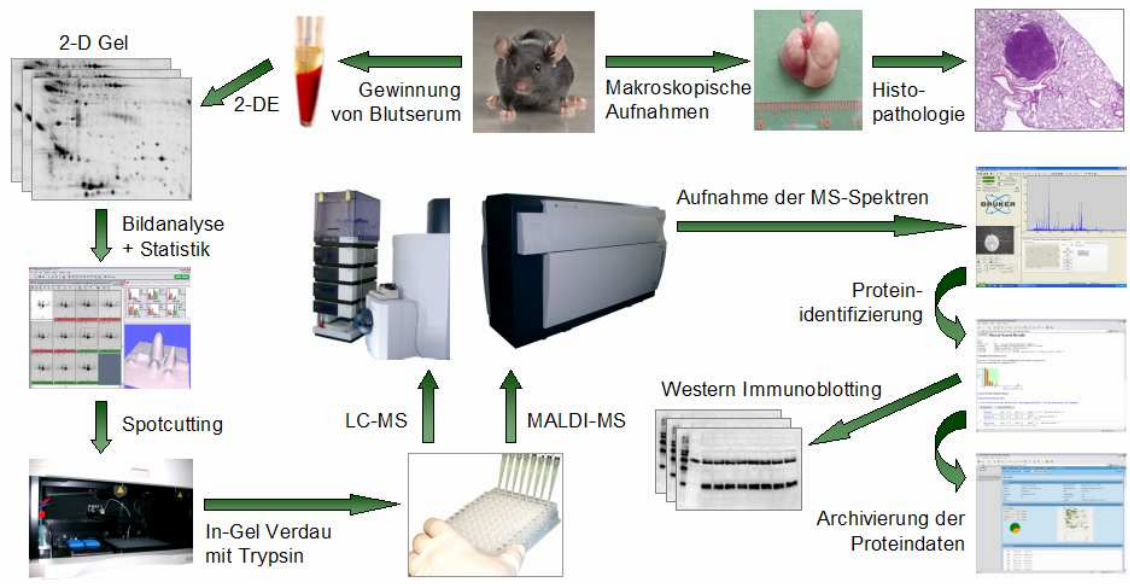


Abb. 2: Arbeitsablauf in der Serumproteomik. Nach Gewinnung von Blutserum aus transgenen Mäusen werden die Proteine mit einem Lysepuffer extrahiert und mittels zweidimensionaler Gelektrophorese (2-DE) aufgetrennt. Die Coomassie-gefärbten Proteinspots werden ausgeschnitten, mit der Endopeptidase Trypsin verdaut und mit Hilfe von MALDI- oder LC-Massenspektrometrie analysiert. Die aufgenommenen Massenspektren werden ausgewertet, Proteine identifiziert, in einer Datenbank archiviert und somit Serumproteomkarten angefertigt. Parallel werden mittels elektronischer Bildanalyseverfahren regulierte krankheitsassoziierte Proteine identifiziert. Quelle (modifiziert): Chatterji B, Ritorto MS, Garaguso I, Belau E, Gazzana G, Borlak J. Onkoproteomics von Lungentumoren. Biospektrum 2006, 7, 732-735.

Ergebnisse aus der Publikation I:
„Serum proteomics of lung adenocarcinomas induced by targeted overexpression of c-raf in alveolar epithelium identifies candidate biomarkers" (Chatterji B, Borlak J. Proteomics 2007, 7, 3980-3991)

Im SP-C/c-raf Mausmodell wurden histopathologisch innerhalb der ersten zwei Lebensmonate morphologische Veränderungen der Lunge in Form einer atypischen adenomatösen Hyperplasie (AAH) beobachtet. Makroskopisch waren erste Tumorfoci bereits nach vier Monaten sichtbar, während im Alter von zwölf Monaten die gesamte Lunge tumorös war (Abb. 3). Serumproteome von Früh- (1 Monat) und Spätstadien (12 Monate) wurden miteinander verglichen. Durchschnittlich wurden 400 Proteinspots pro Gel analysiert. 45 gemeinsame und fünf *de novo* exprimierte Proteine wurden in gesunden nicht-transgenen und transgenen Mäusen identifiziert. Neben diesen *de novo* exprimierten Proteinen waren bei einem Vergleich von Kontrolltieren mit erkrankten Tieren neun Proteine differenziell exprimiert. Diese Proteine werden den Familien der Serpine, Proteaseinhibitoren, Lipocaline, Transthyretine, Globine und Immunoglobuline zugeordnet. Insbesondere wurde im Serum transgener Mäuse ein signifikanter, bis zu 8-facher Anstieg von α-1-Antitrypsin (A1AT), α-2-Macroglobulin (A2M), α-Globin, Major urinary protein (MUP) und Transthyretin (TTHY) beobachtet, die zwar im Zusammenhang mit Lungen- oder Prostatakarzinomen bereits diskutiert werden; die Regulation des Vitamin D-binding protein (VTDB) in Lungentumoren wurde in dieser Doktorarbeit jedoch erstmalig beschrieben.

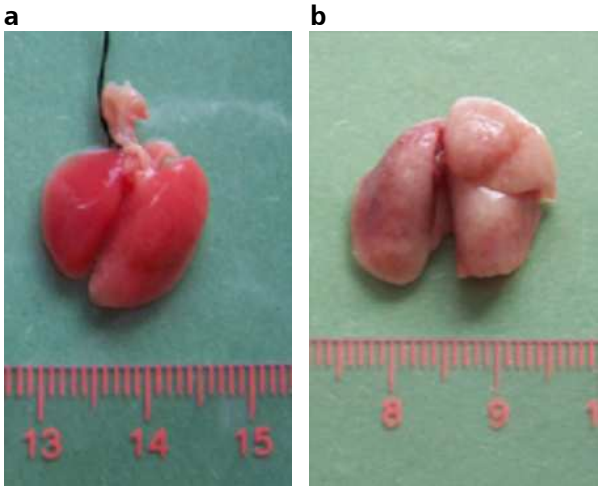


Abb. 3: Makroskopische Darstellung von Lungentumoren in transgenen Mäusen. Im Vergleich zu einer gesunden Lunge (a, nach Perfusion mit PFA 4%) ist eine Lunge mit bronchiolo-alveolärem Karzinom nahezu vollständig durch tumoröses Gewebe durchsetzt (b). Quelle: Chatterji B, Borlak J. A 2-DE MALDI-TOF study to identify disease regulated serum proteins in lung cancer of c-myc transgenic mice. *Proteomics* 2009, *9*, 1044-1056.

Ergebnisse aus der Publikation II:

„A 2-DE MALDI-TOF study to identify disease regulated serum proteins in lung cancer of c-myc transgenic mice" (Chatterji B, Borlak J. Proteomics 2009, 9, 1044-1056)

In SP-C/c-myc transgenen Tieren wurden verschiedene Stadien der Tumorentwicklung beobachtet. Während sich histopathologisch früh im Alveolarepithelium Dysplasien zeigten, wurden nach 8-10 Monaten makroskopisch sichtbare, multifocale bronchiolo-alveoläre Karzinome (BAC) und nach 12-14 Monaten papilläre Adenokarzinome (PLAC) der Lunge (Abb. 4) mit Metastasen in der Leber und anderen Organen beobachtet. Serumproteomanalysen von Früh- (3 Monate) und Spätstadien (14 Monate) führten zu einer Identifikation von 46 unterschiedlichen Proteinen, von denen neun in beiden Stadien statistisch signifikant ($p<0,05$) reguliert waren. Orosomucoid-8, A2M, Apolipoprotein A-I (ApoA1), Apolipoprotein C-III (ApoC3), Glutathione peroxidase 3 (GPX3), Plasma retinol-binding protein (RETBP) und TTHY zeigten erhöhte Expressionen im Serum, während Apolipoprotein E (ApoE) in Spätstadien reprimiert war. Serum amyloid P component (SAP) war ausschließlich in Spätstadien transgener Mäuse exprimiert. Die Expression von ApoA1, RETBP, ApoE und SAP wurde mittels Western Immunoblotting validiert.

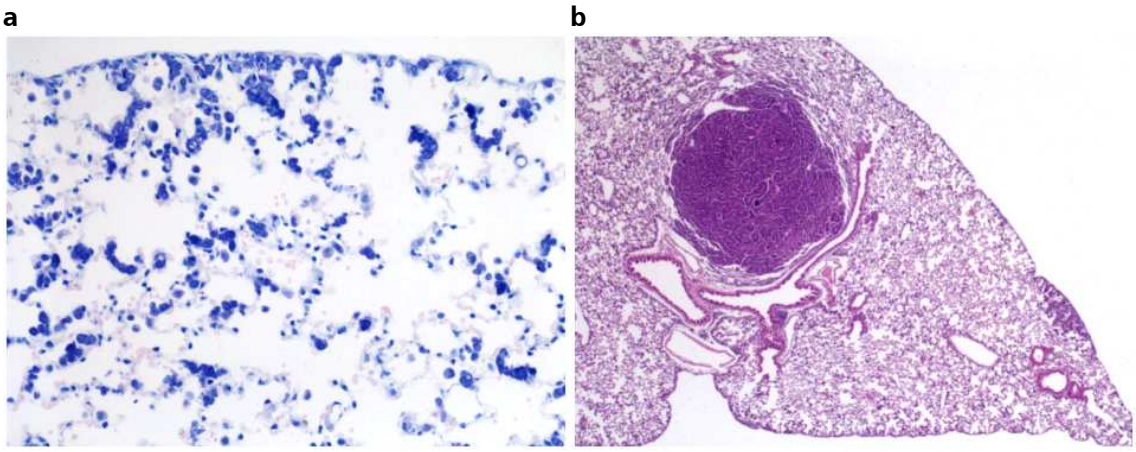


Abb. 4: Histopathologische Darstellung von Lungentumoren in SP-C/c-myc transgenen Mäusen. Lungentumore können anhand histopathologischer Befunde klassifiziert werden (Hematoxylin/Eosin-Färbung). Lunge einer gesunden Maus (a) und einer an Lungenkrebs (PLAC) erkrankten Maus im Alter von 14 Monaten (b). Quelle: Chatterji B, Borlak J. A 2-DE MALDI-TOF study to identify disease regulated serum proteins in lung cancer of c-myc transgenic mice. *Proteomics* 2009, *9*, 1044-1056.

Darüber hinaus wurde in den Promoterregionen der kodierenden Gene einiger krankheitsregulierter Proteine die E-Box Konsensussequenz 5'-CACGTG-3' nachgewiesen, womit ein Zusammenhang mit der direkten Regulation durch den Transkriptionsfaktor c-myc postuliert wird. Im Vergleich zum c-raf transgenen Tiermodell (Publikation I) waren die Proteinexpressionen von A2M, A1AT, Properdin und TTHY vergleichbar. Die Expression von ApoE, ApoA1, ApoC3, GPX3, Orosomucoid-8, RETBP und SAP sind hingegen c-myc spezifisch. Daher sind die regulierten Proteine möglicherweise zur Unterscheidung von AAH und BAC/PLAC besonders relevant. Darüber hinaus spielt ApoE (Abb. 5) eine wichtige Rolle in der Apoptose. Die Regulation von ApoC3 und ApoE wurde in der vorliegenden Doktorarbeit erstmals für Lungentumore beschrieben.

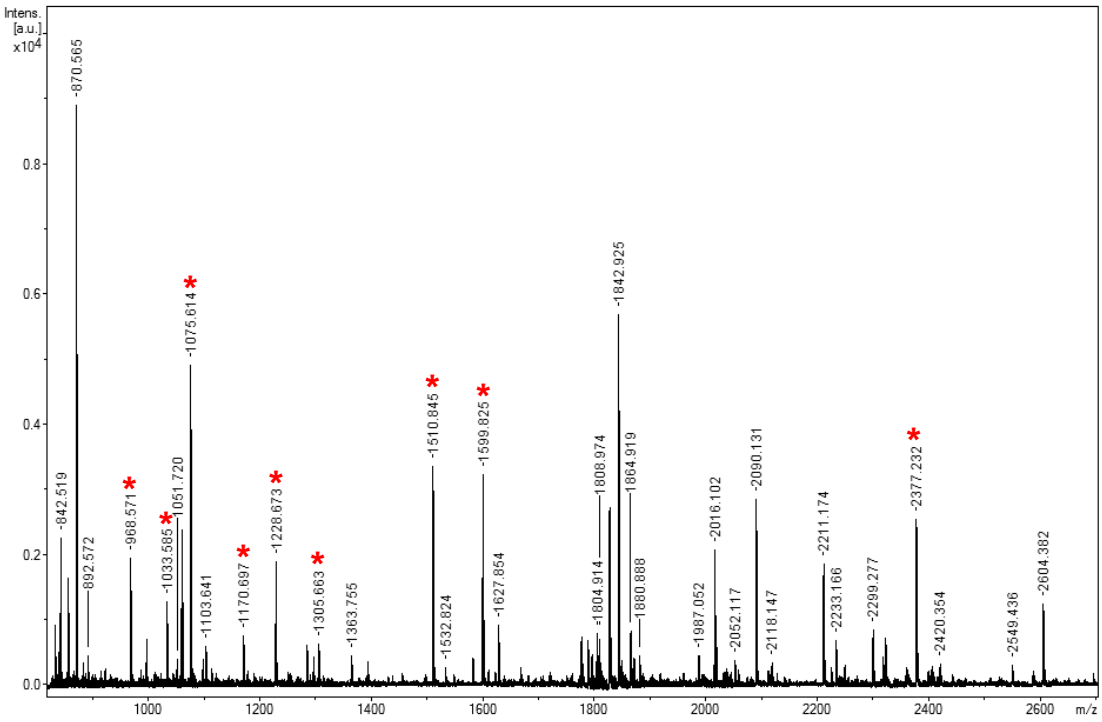


Abb. 5: Massenspektrum von Apolipoprotein E. Ein durch Matrixunterstützte Laserdesorptions-/ionisations-Massenspektrometrie (MALDI-TOF) generiertes Massenspektrum, das zur Identifizierung (*) des Proteins Apolipoprotein E führte. Die Abszisse bezeichnet das Masse/Ladungsverhältnis (m/z), die Ordinate bezeichnet die relative Signalintensität der Ionen in arbitrary units (a.u.).

Ergebnisse aus der Publikation III:
„Search for novel serum biomarkers of dysplasia in a transgenic mouse model of lung cancer" (zur Publikation eingereicht)

Das SP-C/IgEGF Mausmodell zeigte histopathologisch nach 12-14 Monaten Dysplasien im respiratorischen Epithel. Anhand der Histopathologie wurden nach 18-20 Monaten Lungentumore beobachtet (Abb. 6). 68 Proteine einschließlich einiger Isoformen konnten elektrophoretisch zweidimensional aufgetrennt und mittels MALDI-Massenspektrometrie identifiziert werden. 13 Proteine, darunter Transporter, Signalmoleküle und Apolipoproteine, waren statistisch signifikant ($p<0,05$) reguliert. Western Immunoblotting einzelner Serumproteinextrakte transgener Tiere ermöglichte den Nachweis für eine *de novo* Expression von EGF und Amphiregulin; das sind Liganden der EGFR-Tyrosinkinase. Eine Hochregulation in Tieren mit Dysplasien wurde ebenfalls für A2M, ApoA1, Fetuin B, Gelsolin, Vacuolar protein sorting associated protein 28 homologue (VPS28) und Vitamin D-binding protein (VTDB) beobachtet. Dagegen waren Apolipoprotein A-IV (ApoA4), Apolipoprotein M (ApoM), Apolipoprotein H (ApoH), A-raf, Plasminogen und TTHY in transgenen Mäusen reprimiert. In Spätstadien der Tumorgenese sind ebenfalls A2M, ApoA1, ApoA4, ApoM sowie GPX3 signifikant reguliert. Die Ergebnisse der 2-DE konnten für ApoA1, ApoM, A-raf und TTHY ebenfalls mittels Western Blotting bestätigt werden. Der Vergleich mit publizierten Serumproteomstudien der c-raf und c-myc transgenen Tiere (Publikation I + II) zeigt, dass A2M und TTHY in allen drei Mausmodellen reguliert und damit als universelle Markerproteine für die Diagnostik von Lungentumoren von Bedeutung sind. Die Regulation der Apolipoproteine ApoA4 und ApoM wurde für die Entstehung von Lungentumoren im Rahmen dieser Doktorarbeit erstmalig beobachtet. Die Bedeutung dieser Proteine als Biomarkerkandidaten für die Früherkennung von Lungenkrebs ist daher von beträchtlichem Interesse für deren klinische Evaluierung.

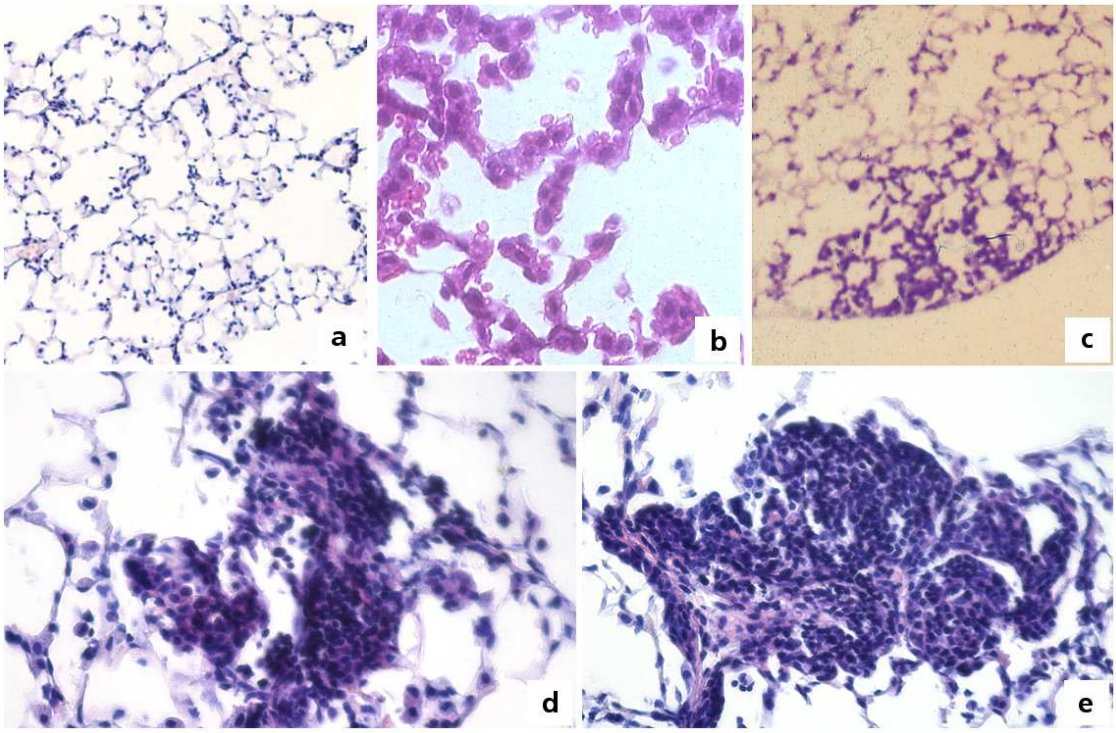


Abb. 6: Histopathologische Darstellung von gesundem und entartetem Lungengewebe. Gesundes Lungengewebe (a), Hyperplasien (b), Dysplasien (c), Adenokarzinome (d und e). Quelle: Chatterji B, Borlak J. Search for novel serum biomarkers of dysplasia in a transgenic mouse model of lung cancer. Submitted for publication, 2009.

Diskussion

Die untersuchten transgenen Modelle zeigten unterschiedliche Stadien der Lungentumorgenese, die makroskopisch und mikroskopisch nachgewiesen wurden. In dieser Doktorarbeit wurden die Serumproteome der Mausmodelle SP-C/c-raf, SP-C/c-myc und SP-C/IgEGF erstmalig untersucht und kartiert. Mittels zweidimensionaler Gelelektrophorese und MALDI-MS/MS wurde basierend auf >25.000 In-gel Verdaus eine Proteomdatenbank mit 88 gemeinsamen Serumproteinen aufgebaut, die als Referenz für weitere Studien mit transgenen Mausmodellen dient und für die genaue Zusammensetzung ihrer Serumproteome aufschlussreich sein kann.

Mit Hilfe softwarebasierter Bildanalyseverfahren wurden Serumproteome in Form von nahezu dreihundert 2-D Gelen von gesunden Kontrolltieren und transgenen Tieren miteinander verglichen („differential display“) und regulierte Proteinspots detektiert, die sich als Biomarkerkandidaten für die Erkennung von Lungentumoren eignen können. Gegenwärtig sind nur wenige Biomarker zur Erkennung von SCLC und NSCLC bekannt, darunter CEA (carcinoembryonic antigen), CYFRA 21-1 (cytokeratin 19 fragment), TPA (tissue polypeptide antigen), ProGRP (progastrin-releasing peptide), NSE (neuron-specific enolase) und Tumor M2-pyruvate kinase. Viel diskutierte Markerproteine sind Serum amyloid A, haptoglobin-α 2, ApoA1 und KLKB1 (plasma kallikrein B1 fragment) [Sung HJ *et al. BMB Rep.* 2008, *41*, 615-625].

Die Ergebnisse dieser Doktorarbeit liefern zum einen Hinweise für universelle Marker wie die Akute-Phase-Proteine TTHY und A2M, die sowohl in Früh- als auch Spätstadien der Lungentumorgenese, d.h. AAH bzw. BAC/PLAC reguliert waren. Deren Regulation wurde bereits in Patienten mit Bronchial-, Ovarial- und Prostatakarzinomen beobachtet und ist daher krankheitsrelevant. Zum anderen wurden selektive Marker zur Unterscheidung von AAH und BAC/PLAC mittels Serumproteomik identifiziert, deren Regulation für BAC/PLAC spezifisch waren und bisher noch nicht berichtet wurden, darunter ApoE, ApoC3 und ApoH. Im

Gegensatz dazu sind MUP, VTDB und EGFR, die selektiv in AAH reguliert oder *de novo* exprimiert wurden, mögliche prädiktive Marker für die Erkennung von Lungentumoren des Menschen. Insbesondere sind eine Reihe von Apolipoproteinen wie ApoM, ApoA4 und ApoE von großer klinischer Bedeutung und können als potenzielle Biomarker vorgeschlagen werden.

Die Findung neuer Biomarker zur Erkennung von Lungentumoren sowie die Unterscheidung verschiedener Tumorsubtypen wurde zum Patent angemeldet (Borlak J and Chatterji B).

Ausblick

Basierend auf den Ergebnissen dieser Doktorarbeit ist die Translation in die Klinik das nächste Ziel. Da die Entnahme von Patientenblut und Gewinnung von Serum und anderen Körperflüssigkeiten wie Sputum etc. ohne großen Aufwand realisierbar ist, sind klinische Studien zur Entwicklung diagnostischer Kits mit evidenzbasierten Biomarkern der nächste Schritt zur Früherkennung von Lungenkrebs des Menschen. Zudem ist eine Unterscheidung verschiedener Arten von Lungentumoren anhand von Serummarkern für eine gezielte Krebstherapie von besonderer Relevanz. Trotz gesteigerter Überlebenschancen durch Vorsorgeuntersuchungen von Patienten mit Bronchialkarzinomen, ließe sich mittels standardisierten immunologischen Bluttests auf Basis spezifischer Biomarker eine frühzeitige Diagnose von Tumoren stellen. Ist ein Tumor in der jeweiligen Krankheitsprogression nicht mehr heilbar, sind Tumormarker zudem als Verlaufskontrolle in der palliativen Therapie für den Patienten und den behandelnden Arzt von großem Nutzen.

Publikation I:

Serum proteomics of lung adenocarcinomas induced by targeted overexpression of c-raf in alveolar epithelium identifies candidate biomarkers

Published in: *Proteomics* 2007, 7, 3980-3991

Weblink: http://www3.interscience.wiley.com/journal/116324641/abstract

Bijon Chatterji [1], Jürgen Borlak [1,2]

[1] Department of Drug Research and Medical Biotechnology, Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany
[2] Center for Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

Correspondence:

Prof. Dr. J. Borlak
Department of Drug Research and Medical Biotechnology
Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM)
Nikolai-Fuchs-Str. 1
D-30625 Hannover, Germany
Phone: +49-511-5350-559
Fax: +49-511-5350-573
E-mail: borlak@item.fraunhofer.de

Abbreviations:

c-raf, serine-threonine kinase of the Raf family; **NSCLC,** non-small cell lung carcinomas; **ras,** rat sarcoma; **SCLC,** small cell lung carcinomas; **SP-C,** surfactant protein C

Keywords: Serum / c-raf / Lung adenocarcinoma / Biomarkers / 2-DE

Abstract

We previously reported a proteome map of lung adenocarcinomas in c-raf transgenic mice. We now extend our initial studies to serum proteins at early stage (1 month) and advanced stages of tumorigenesis (12 months). Notably, serum proteins from wildtype and tumor bearing mice were extracted with a lysis buffer containing 5 mol/L urea, 2 mol/L thiourea, 40 mmol/L Tris, 4% CHAPS, 100 mmol/L DTT, 0.5% BioLyte 3-10, separated by 2-DE and studied by image analysis. On average 400 protein spots per gel were excised and analyzed by MALDI-TOF MS. We identified 45 common and 5 uniquely expressed proteins in wildtype and tumor bearing mice. Apart from uniquely identified proteins we observed for n=9 proteins differential expression when wildtype and tumor bearing mice were compared. This included serpins and other protease inhibitors, lipocalins, transthyretins, globins and immunoglobulins. Notably, we demonstrate significant regulation of alpha-1-antitrypsin, alpha-2-macroglobulin, hemoglobin subunit alpha, vitamin D-binding protein, major urinary proteins and transthyretin (up to 8-fold) in serum of lung tumor bearing mice. Disease association of these proteins in human malignancies has been reported. Thus, an identification of regulated serum proteins in this lung cancer disease model provides excellent opportunities for the search of novel biomarkers.

1 Introduction

In 2002, an estimated 1.2 million new cases of death caused by lung cancer were diagnosed worldwide (American Cancer Society, 2002). Smoking is considered as the major risk factor and accounts for > 80% of all diagnosed cases [1]. Other risk factors include inhalation of radioactive compounds, heavy metals, asbestos and petrochemicals [2].

In general, lung tumors are classified by histological phenotypes and are divided into small cell lung carcinomas (SCLC) and non-small cell lung carcinomas (NSCLC). The latter group is further divided into adenocarcinoma, large cell carcinoma and squamous cell carcinoma. Notably, classification of human lung carcinomas by mRNA expression profiling revealed distinct subclasses of adenocarcinomas that might arise from bronchial, Clara and alveolar epithelium [3]. Recent data suggest a significant rise in alveolar malignancies and may account for as many as 30% of all adenocarcinomas [4].

One of the molecular causes leading to NSCLC has been linked to enhanced mitogen activated kinase signalling of the ras-raf cascade resulting in high capacity cell division and lung tumor formation [4]. Note, raf is an essential serine/threonine kinase constituent of the MAPK signalling pathway and a downstream effector of the central signal transduction mediator ras. Both, ras and raf are encoded by proto-oncogenes which become oncogenes when mutated. The MAPK pathway is dysregulated in a remarkable proportion of human malignancies through aberant signalling upstream of the protein and by activating mutations of the protein itself, both of which confer a proliferative advantage. Therapeutics targeting c-raf in NSCLC and SCLC have therefore been evaluated [5, 6].

We are specifically interested in the role of c-raf in lung cancer biology and therefore studied disease onset and progression in a transgenic mouse model [5]. These mice overexpress an activated form of c-raf-1, which mimics the effect of c-ras activation [7]. The transgene was targeted to alveolar epithelial cells through use of the surfactant protein C promotor (SP-C). In transgenic mice, the first

morphological changes occurred in distinct areas of the lung within the first 2 months. After eight to ten months the whole lung was morphologically changed showing typical features of lung adenocarcinomas [5].

At present, the tumor proteome of c-raf-induced lung adenocarcinomas is unknown. Disease proteomics, however, may provide new insights into the molecular events associated with lung carcinogenesis. Previous works from our laboratory aimed at identifying pulmonary proteins expressed in lung tissue of c-raf-induced lung tumors and of differentially expressed proteins for their putative value in diagnostics and therapy [8]. We now extend our initial findings to the serum proteome of c-raf transgenic mice and report *de novo* expression of tumor associated proteins and regulation of serum proteins.

2 Materials and methods

2.1 Biological material and protein extraction

2.1.1 SP-C/c-raf model

SP-C/c-raf transgenic lung tumor bearing mice were obtained from the laboratory of Prof. Ulf Rapp (University of Würzburg, Germany). A detailed description of the SP-C/c-raf transgenic line is given in [7] and [8]. Blood serum of tumor bearing mice (n=6, aged 1 month and n=15, aged 12 months) and blood serum of wildtype mice (n=4, aged 1 month and n=12, aged 12 months), that served as controls, were studied. Lung tumors arose multifocally. Starting as adenomatous hyperplasia (AAH), first morphological changes in distinct areas of the lung appeared within the first 2 months, only visible through histopathology. Macroscopically, tumors were visible after 4 months. For our studies we used 1 month old mice at an early stage of tumor development, and 12 months old mice with advanced tumor growth. At this point (12 months) almost the entire lung is tumorous (see Fig. 5 for histopathology). Hematoxylin and eosin staining

were used for histopathology of tumors. We compared expression of pulmonary proteins of tumor bearing with those of wildtype mice.

2.1.2 Serum sample preparation

Blood serum was collected from the *vena cava* and allowed to clot for 2 h at room temperature. The clotted material was removed by centrifugation at 3000 rpm for 15 min. Hemolysis was not observed. The sera obtained from the blood samples were frozen immediately without any further treatment in liquid nitrogen and stored at -80°C until further analysis. The protein concentration of serum was determined by the Bradford protein assay (Protein Assay Dye Reagent Concentrate, Bio-Rad), using bovine gamma globulin as the standard. Extraction yields ranged from 80 to 90 µg/µL for both wildtype and tumor samples.

2.2 2-DE

2.2.1 IEF

In the first dimension, proteins were separated by IEF with precast IPG strips (pH 3-10, non-linear gradient and pH 4-7, linear gradient; both 170x3x0.5 mm, Bio-Rad). Of the total proteins, 1 mg was diluted in a lysis buffer (5 mol/L urea, 2 mol/L thiourea, 40 mmol/L Tris, 4% CHAPS, 100 mmol/L DTT, 0.5% BioLyte 3-10; Bio-Rad) to obtain a total volume of 350 µL per strip. Focused IPG strips were rehydrated at 50 V for 12 h. IEF was performed at 20°C with a maximum voltage of 10 kV and a maximum current of 50 µA per strip. Each sample was analyzed in triplicate. After IEF, IPG strips were stored at -80°C until SDS-PAGE.

2.2.2 Reduction and alkylation

After IEF, IPG strips were equilibrated in 10 mL reducing buffer (2% DTT in 10 mL equilibration buffer containing 6 mol/L urea, 30% glycerin, 2% SDS, 0.05 mol/L

Tris-HCl, pH 8.8) for 15 min, followed by 15-min equilibration in 10 mL alkylation buffer (400 mg iodoacetamide in 10 mL equilibration buffer).

2.2.3 SDS-PAGE

SDS-PAGE was performed in a Protean-plus Dodeca™ Cell (Bio-Rad) using self-cast polyacrylamide gels (200 x 205 x 1.5 mm; 12% T). Gels were run in parallel in 0.025 mol/L Tris/ 0.192 mol/L glycine/ 0.1% SDS at 10°C with a constant voltage of 70 V. The 2-DE standards (Bio-Rad) used for M_r and p*I* calibration were as follows: hen egg conalbumin type I (76 kDa; p*I* 6.0, 6.3, 6.6); BSA (66.2 kDa; p*I* 5.4, 5.6); bovine muscle actin (43 kDa; p*I* 5.0, 5.1); rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36 kDa; p*I* 8.3, 8.5); bovine carbonic anhydrase (31 kDa, p*I* 5.9, 6.0); soybean trypsin inhibitor (21.5 kDa; p*I* 4.5); and equine myoglobin (17.5 kDa; p*I* 7.0).

2.3 Protein staining

Gels were fixed overnight in 500 mL 30% ethanol/ 2% phosphoric acid, washed three times for 20 min each in 500 mL 2% phosphoric acid and equilibrated with 500 mL 2% phosphoric acid/ 18% ethanol/ 15% ammonium sulfate. Colloidal CBB staining of proteins was initiated by addition of 6 mL staining solution (2% CBB G250, Roth) to 500 mL of equilibration solution. Gels were stained for 48 h and thereafter washed once with 500 mL water for 20 min.

2.4 Image analysis

Gel images were scanned with the Molecular Imager Pharos FX (Bio-Rad). Spot detection, quantification and comparison of 2-D protein patterns was done with the PDQuest 8.0 software (Bio-Rad). Background and vertical streaks were removed from each gel image and spots were digitized by Gaussian fit. For quantification a "matchset" of all gels was prepared and the absorbance of individual protein spots from 2-D gels was assessed. The raw quantity of each spot

in a member gel was divided by the total intensity value of all the pixels in the image (i.e., total density in gel image); this normalization procedure of the PDQuest program assumed that the total density of an image (background density plus spot density) will be relatively consistent from gel to gel. After generation of the analysis sets, the selected spots were excised and transferred to 96-well microtiter plates (ABgene) by the EXQuest spot cutter (Bio-Rad).

2.5 Protein identification by MALDI MS

2.5.1 In-gel digestion

Each of the CBB-stained gel plugs was dehydrated in 50 µL acetonitrile (ACN), rehydrated/washed with 50 µL ammonium hydrogencarbonate solution (50 mmol/L) and then dehydrated with 50 µL ACN. Digestion with 20 ng/µL trypsin (Sequencing grade, Promega) was performed at 37°C for 4 h. Resulting peptides were extracted with 5 µL 1% TFA in an ultrasonic bath (Sonorex, Super RK 514 BH, Bandelin).

2.5.2 MALDI-TOF analysis

Extracted peptides were spotted directly onto a 600 µm/384 well AnchorChip sample target (Bruker Daltonics) using the affinity preparation protocol recommended by the manufacturer (matrix: CHCA). The MALDI mass spectra were obtained using a Bruker Ultraflex II TOF/TOF mass spectrometer equipped with a 384-sample scout source (Bruker Daltonics). An external peptide calibration standard containing the following fragments was used to calibrate the instrument: angiotensin II ($[M+H]^+$ 1046.54); angiotensin I ($[M+H]^+$ 1296.68); substance P ($[M+H]^+$ 1347.74); bombesin ($[M+H]^+$ 1619.82); ACTH clip 1–17 ($[M+H]^+$ 2093.09); ACTH clip 18–39 ($[M+H]^+$ 2465.20); somatostatin 28 ($[M+H]^+$ 1347.47) (Bruker Daltonics). Peptide masses were searched against the Swiss-Prot database employing the MASCOT program (in-house MASCOT-server) [9] for protein identification. Database searches were performed taking into account

carbamidomethyl modification of cysteines and possible oxidation of methionine, and allowing one missed cleavage. A mass inaccuracy of <100 ppm was required for PMF. For further consideration, only those proteins were assumed to be identified that were annotated from corresponding spots in at least three gels with a MASCOT score >>53 being clearly separated from the next best match. MS/MS analysis was performed when necessary. Identified proteins were sent to the Proteinscape™ database (Protagen) and checked individually for further consideration.

3 Results and discussion

3.1 Serum proteomics of SP-C/c-raf model mice

As described in the materials and methods, we used a thiourea-containing lysis buffer to extract proteins from serum [10, 11]. Proteins were separated within pH ranges of 3-10 and 4-7 and visualized with the colloidal CBB (CCB) stain. With the CCB stain approximately 400 spots/gel were detected. Figure 1 depicts a serum reference map for wildtype mice (pH 3-10).

3.2 Identification of serum proteins in SP-C/c-raf mice by MS

About 400 spots per gel were excised from CCB-stained gels. Protein spots from three parallel gels of each sample were analyzed by PMF using MALDI-TOF MS after tryptic in-gel digest. Identification was carried out by Swiss-Prot database searches with MASCOT. When needed, protein identification was confirmed by additional MS/MS experiments.

In tumor bearing and wildtype mice, 45 common (Tab. 1) and 5 unique serum proteins were identified by MS and MS/MS. Our identification of serum proteins fits best to those of Duan *et al.* [12], who reported 38 unique proteins and of Wait *et al.* [13] with 28 distinct proteins (Fig. 2). Overall, we identified 24 serum

proteins not reported by the afore-mentioned investigators. Furthermore 8 serum proteins identified in this study are also expressed in lung tissue as reported previously [8] and included A1AT4_MOUSE (spot no. 4), A1AT6_MOUSE (spot no. 5), ACTG_MOUSE (spot no. 8), ALBU_MOUSE (spot no. 10), APOA1_MOUSE (spot no. 11), HBA_MOUSE (spot no. 25), TRFE_MOUSE (spot no. 41) and TTHY_MOUSE (spot no. 42).

3.3 Differential expression of serum proteins in SP-C/c-raf transgenic mice

Nine proteins were found to be differentially expressed (matched in >3 different gels) with a minimum of 2-fold regulation, whereas five proteins were found to be exclusively expressed (see 3.4) when extracts of wildtype and lung tumor serum proteomes (aged 12 months) were compared. Table 2a and 2b and Figure 3a and 3b depict examples of differentially expressed proteins from wildtype and tumor bearing mice. Additionally, extracts from wildtype and tumor bearing mice at an age of 1 month were analyzed to compare expression between early and late stages of tumor development. Seven proteins were regulated in both tumor stages (Tab. 2a). Prominent examples are discussed below in terms of their regulation in human malignancies, overexpression of the c-raf kinase and regulation at different stages of cancerogenesis. Fig. 4 depicts a comparison of average regulation of these proteins between mice, aged 1 month and 12 months.

Spot no. 3 and no. 5 were identified as **alpha-1 antitrypsin** isoforms (A1AT, Swiss-Prot accession no.: Q00896 and P81105; A1AT3_MOUSE and A1AT6_MOUSE). A1AT is a secretory glycoprotein produced mainly in the liver and monocytes. It is the most abundant serine protease inhibitor in human plasma. It predominantly inhibits neutrophil elastase and therefore prevents breakdown of lung tissue. The deficiency of A1AT is an inheritable disorder characterized by reduced serum levels of A1AT. Protease inhibitors Z (PiZ) and protease inhibitors S (PiS) are the most common deficient genotypes of A1AT [14]. In previous studies A1AT was shown to be regulated in lung cancer [15, 16, 17]. Notably, Woodworth

et al. reported induction of the c-raf kinase in hepatocellular carcinoma along with overexpression of A1AT [34]. Likewise, we demonstrate overexpression of A1AT in tumor bearing mice at early (1 month) and advanced stages (12 months) of lung tumor development.

Spot no. 7 was identified by PMF as **alpha-2-macroglobulin** (A2MG, Swiss-Prot accession no.: Q61838; A2MG_MOUSE) from the protease inhibitor I39 family. This plasma-specific protein is able to inhibit all four classes of proteinases by a unique "trapping" mechanism [18]. More than twenty years ago A2MG was reported to be a lung tumor marker being significantly increased in human tumor patients [19]. A2MG levels, however, were slightly increased in patients with pulmonary emphysema and pneumonia as well [15]. In the study of Misra and coworkers incubation of prostate cancer cells with A2MG caused induction of c-raf expression [35]. We observed a > 2-fold upregulation of this protein in tumor bearing mice. Note, A2MG regulation at initial stages of cancerogenesis was repressed or unchanged. We propose alpha-2-macroglobulin as a lung cancer dependent candidate biomarker for advanced stages of disease.

Spot no. 25 was identified as **hemoglobin subunit alpha** (HBA, Swiss-Prot accession no.: P01942; HBA_MOUSE). This protein is one of the subunits of hemoglobin that belongs to the globin superfamily and is a heme-containing protein in the red blood cells, involved in binding and/or transporting oxygen from the lung to various peripheral tissues. Experiments have demonstrated that the expression of HBA was upregulated upon specific apoptotic stimuli like cytokine deprivation or cisplatin treatment in a hematopoietic pro-B cell line [20]. These data indicate that HBA is a new and crucial factor in apoptosis, supporting the mitochondrial pathway. Moreover, HBA was regulated in ovarian cancer [36]. Angiogenic studies with 17-DMAG, an orally bioavailable heat shock protein 90 modulator, decreased hemoglobin levels in vivo and reduced c-raf-1 expression in vitro [37]. In serum of tumor bearing mice, HBA was overexpressed.

Spot no. 36 was identified as a **major urinary protein** (MUP). MUPs belong to

the lipocalin superfamily, bind and slowly release male-specific pheromones in deposited scent marks. Likewise, females also express these proteins, consistent with their role in encoding individual signatures in scent marks [21]. Urinary proteins had been used as biomarkers for lung cancer, such as pseudouridine [22]. In a genomics approach MUP was found to be repressed in lung adenocarcinomas [23]. In our study, however, MUP was upregulated in the serum of tumor bearing mice. Both, at early stage by 6-fold (Swiss-Prot accession no.: P11589; MUP2_MOUSE), and at advanced stages of tumorigenesis by > 4-fold (Swiss-Prot accession no.: P04938; MUP8_MOUSE). A c-raf dependent regulation of MUPs was not reported so far.

Spot no. 42 was identified as **transthyretin**, also known as prealbumin (TTR, Swiss-Prot accession no.: P07309; TTHY_MOUSE). TTR is a regular blood protein which belongs to the same-named superfamily. It carries thyroid hormones, such as thyroxine and tri-iodothyronine from bloodstream to tissues. Furthermore transthyretin facilitates retinol transport through interaction with the **retinol binding protein** (RBP, spot no. 38, Swiss-Prot accession no.: P00724; RETBP_MOUSE). Mice lacking transthyretin expression have dramatically lower levels of retinol and RBP as well as cellular RBP and was shown to be associated with malignant transformation of ovarian surface epithelium [24, 25]. Notably, Zhang *et al.* reported a truncated form of transthyretin to be repressed in human patients with ovarian cancer [26] and suggests its use as a biomarker [27]. A recent proteomic study demonstrated, however, upregulation of transthyretin in human lung adenocarcinomas. In particular, the TTR monomer may be a blood marker for cerebrospinal fluid barrier disruption that occurs, for instance, in cerebral metastasis [28], but might also be regulated in carcinomas [29]. A downregulation of transthyretin by more than 6-fold was reported for ovarian [30] and lung cancer patients. We observed an initial 1.3-fold upregulation of this protein at an early stage of lung cancer (1 month), but a more than four-fold downregulation in mouse serum at advanced stages of tumor growth (12 months). The role of c-raf in the regulation of transthyretin is uncertain. Nonetheless, results from the present study and those reported by others point to TTR as an interesting

candidate biomarker.

Based on PMF, spot no. 43 was identified as **vitamin D-binding protein** (DBP, Swiss-Prot accession no.: P21614; VTDB_MOUSE) and regulated in serum of tumor bearing mice. This protein belongs to the ALB/AFB/VDB family and can be found in plasma, ascitic fluid, cerebrospinal fluid, urine and on the surface of many cell types. In plasma, this multifunctional protein acts as a vitamin D sterol carrier and binds the actin monomers, thereby preventing its polymerization. DBP associates with membrane-bound immunoglobulin on the surface of B-lymphocytes, furthermore with membrane-bound IgG Fc receptor of T-lymphocytes [31]. Deglycosylation of serum DBP led to immunesuppression in cancer patients [32]. According to Hlavaty *et al.* DBP may be of utility as a serum biomarker in the early detection of prostate cancer [33]. > 2-fold upregulation of DBP at advanced stages of lung cancerogenesis is a novel finding. Note, 1,25-dihydroxyvitamin D3, a vitamin D3 metabolite, caused activation of c-raf in human keratinocytes [38]. Additionally, in our studies DBP was downregulated by 1.3-fold in serum of lung tumor bearing mice at early stage of tumor growth (1 month). These findings warrant an evaluation of DBP as a candidate biomarker in cancer patients.

3.4 Exclusive expression of serum proteins in SP-C/c-raf transgenic mice

Notably, five proteins were found to be exclusively expressed either in wildtype or tumor bearing mice, aged 12 months, which are discussed below.

A soluble form of the **epidermal growth factor receptor** (EGFR, spot no. 46, see Tab. 2b, Swiss-Prot accession no.: Q01279; EGFR_MOUSE) was exclusively identified in serum of wildtype mice. EGFR is a membrane-bound tyrosine kinase, but soluble forms were also found in body fluids. Binding of EGF to EGFR leads to dimerization and internalization of the EGFR complex. Through phosporylation of tyrosine residues the EGFR connects to the MAPK/ERK pathway, with c-raf as a key member, thereby stimulating DNA synthesis and cell proliferation. As reviewed by Mendelsohn *et al.*, the EGFR is a suitable target in the drug treatment of cancers.

EGFR is overexpressed, dysregulated or mutated in many epithelial malignancies, and activation of EGFR appears important in tumor growth and progression [39]. EGFR was proposed as a serum biomarker in breast and ovarian cancer [40]. Studies on human sera by Baron and coworkers demonstrated repression of sEGFR in ovarian cancer [41, 42, 43].

The role of serum **properdin** (spot no. 50, see Tab. 2b, Swiss-Prot accession no.: P11680; PROP_MOUSE) in tumor bearing mice was investigated by Schwartz *et al.* more than fourty years ago [44, 45]. In our studies, properdin was found exclusively expressed in wildtype mice. Properdin, also known as factor P, is a positive regulator of the alternate pathway of complement. It binds to and stabilizes the C3- and C5-convertase enzyme complexes. It is known that properdin participates in some specific immune responses. It plays a part in tissue inflammation as well as in the engulfing of pathogens by phagocytes. In addition, properdin is known to support neutralization of some viruses. As a component of the alternative pathway for complement activation, properdin complexes with another protein, C3b, to stabilize the alternative C3 convertase (C3bBb).

The **immunglobulin gamma-2B chain C region** (spot no. 47, see Tab. 2b, Swiss-Prot accession no.: P01867; GCBM_MOUSE) was found to be exclusively expressed in wildtype mice, whereas the **immunglobulin J chain** (spot no. 48, see Tab. 2b, Swiss-Prot accession no.: P01592; IGJ_MOUSE) and **immunglobulin kappa chain V-III region MOPC 70** (spot no. 49, see Tab. 2b, Swiss-Prot accession no.: P01656; KV3C_MOUSE) were found exclusively expressed in tumor bearing mice. To the best of our knowledge, their role in lung cancer is unknown.

4 Concluding remarks

Based on the initial works of our laboratory [8] we now extended our investigations to the serum proteome of lung tumor bearing mice. Two stages of tumor development were studied (1 month and 12 months). Protein expression of tumor bearing mice was compared with those of wildtype, e.g. non-transgenic animals. In total, 8 proteins identified in tissue of lung tumor bearing mice were also identified in serum. A total of 50 proteins were identified in serum, some of which were specifically regulated or exclusively expressed either in tumor bearing or wildtype mice. The biological functions of the identified proteins associated with tumorigenesis are not always clear. Notably, we found alpha-1-antitrypsin (A1AT) and alpha-2-macroglobulin (A2MG) to be upregulated in the serum proteome of 12 months old mice and according to literature its regulation is linked to c-raf overexpression [34, 35]. We extend early findings for their regulation in tissue [15, 16]. A1AT was upregulated in 1 month old mice as well, whereas expression of A2MG was downregulated in 1 month old mice. Hemoglobin subunit alpha was upregulated in serum samples of lung tumor bearing mice (12 months) and may play a role in apoptosis [20]. We found major urinary proteins to be upregulated both, in early and late stages of tumor development. MUPs may serve as biomarkers for lung cancer [12, 22]. Upregulation of vitamin D-binding protein (DBP) in advanced stages of lung cancer is a novel finding. A role for c-raf activation by a vitamin D metabolite in human keratinocytes has already been reported [38]. We found expression of transthyretin to be upregulated in 1 month and repressed in 12 month old tumor bearing mice. Therefore, previously reported findings fit well with our results of regulated serum proteins. We thus propose their in-depth validation as biomarker candidates for the detection of lung adenocarcinomas.

Figure captions

Figure 1: 2-D reference map of blood serum from wildtype mice stained with CBB.

Figure 2: Comparison of works investigating the mouse serum proteome. Duan and coworkers reported 38 mouse serum proteins, while Wait *et al.* reported 28 proteins. We identified 50 proteins, 24 of them were novel and not reported so far. 14 proteins were in common.

Figure 3a/b: Examples of differentially expressed serum proteins of tumor bearing (T) and wildtype (C) mice. Protein spots of interest are marked by circles and/or arrows. **a:** differentially expressed serum proteins from mice, aged 1 month. **b:** differentially expressed serum proteins from mice, aged 12 months.

Figure 4: Comparison of protein expression profiles. Comparison of expression profiles of regulated serum proteins between lung tumor bearing and wildtype mice (1 month and 12 months). T/C: average ratio between n tumor bearing (T) and n wildtype (C) mice. T/C = 1.0: no regulation, T/C < 1.0: proteins are downregulated in tumor bearing mice, T/C > 1.0: proteins are upregulated in tumor bearing mice.

Figure 5: Histopathology of tumorigenesis in the lung of c-raf transgenic mice. Lung tumors arose multifocally. Starting as adenomatous hyperplasia (AAH), first morphological changes in distinct areas of the lung appeared within the first 2 months. This figure shows advanced stages of tumorigenesis (12 months), when almost the entire lung is tumorous.

Table captions

Table 1: **Protein identification in 2-DE maps of serum proteins from wildtype and SP-C/c-raf transgenic lung tumor bearing mice, identified by MALDI-MS.** See supplementary table 1 for detailed information.

Table 2a/b: **Quantification of regulated proteins from 2-D gels. a:** Protein expression profiles from tumor bearing (T) and wildtype (C) mice, aged 1 month. **b:** Protein expression profiles from tumor bearing (T) and wildtype (C) mice, aged 12 months. Significant differential expression of protein spots that were quantified from 2-D gels. Quantification of protein abundance was done using the PDQuest 2-D software (Bio-Rad) by measuring the normalized OD (arbitrary units, AU) of each protein spot. The change in abundance of the proteins is expressed by the calculated ratio between average tumor (T) and wildtype (C) protein expression values. Exclusively expressed proteins are marked by T or C.

Supplementary Table 1: **An overview of mouse serum proteins.** The Mascot score, the number of identified peptides, their sequence and the protein coverage of the best hits are shown for each identified protein. O@M: Abbreviation for oxidation at the amino acid methionine.

The authors have declared no conflict of interest.

5 References

[1] Hirsch FR, Bunn PA, Dmitrovsky E, Field JK *et al.* IV international conference on prevention and early detection of lung cancer, Reykjavik, Iceland, August 9-12, 2001. *Lung Cancer* 2002, 37, 325–344.

[2] Christiani DC. Smoking and the molecular epidemiology of lung cancer. *Clin Chest Med* 2000, 21, 87–93.

[3] Bhattacharjee A, Richards WG, Staunton J, Li C *et al.* Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci USA* 2001, 98, 13790–13795.

[4] Takezawa C, Takahashi H, Fujishima T, Shiratori M *et al.*, Assessment of differentiation in adenocarcinoma cells from pleural effusion by peripheral airway cell markers and their diagnostic values. *Lung Cancer* 2002, 38, 273–281.

[5] Kerkhoff E, Fedorov LM, Siefken R, Walter AO *et al.* Lung-targeted expression of the c-Raf-1 kinase in transgenic mice exposes a novel oncogenic character of the wild-type protein. *Cell Growth Differ* 2000, 11, 185-190.

[6] Graziano SL, Pfeifer AM, Testa JR, Mark GE *et al.* Involvement of the RAF1 locus, at band 3p25, in the 3p deletion of small-cell lung cancer. *Genes Chromosomes Cancer* 1991, 3, 283-293.

[7] Bonner TI, Oppermann H, Seeburg P, Kerby SB *et al.* The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene. *Nucleic Acids Res* 1986, 14, 1009-1015.

[8] Rütters H, Zürbig P, Halter R, Borlak J. Towards a lung adenocarcinoma proteome map: studies with SP-C/c-raf transgenic mice. *Proteomics* 2006, 6, 3127-3137.

[9] Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999, 20, 3551–3567.

[10] Lehner I, Niehof M, Borlak J. An optimized method for the isolation and identification of membrane proteins. *Electrophoresis* 2003, 24, 1795–1808.

[11] Rabilloud T. Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. *Electrophoresis* 1998, 19, 758–760.

[12] Duan X, Yarmush DM, Berthiaume F, Jayaraman A, Yarmush ML. A mouse serum two-dimensional gel map: application to profiling burn injury and infection. *Electrophoresis* 2004, 25, 3055-3065.

[13] Wait R, Chiesa G, Parolini C, Miller I *et al.* Reference maps of mouse serum acute-phase proteins: changes with LPS-induced inflammation and apolipoprotein A-I and A-II transgenes. *Proteomics* 2005, 5, 4245-4253.

[14] El-Akawi ZJ, Nusier MK, Zoughool FE. Relationship between alpha-1 antitrypsin deficient genotypes S and Z and lung cancer in Jordanian lung cancer patients. *Saudi Med J* 2006, 27, 181-184.

[15] Umeki S, Niki Y, Soejima R. Elastase/antielastase systems in pulmonary diseases. *Am J Med Sci* 1988, 296, 103-106.

[16] Di Martino G, Iannucci F, Bizzarro A, Iacono G. Association of serum tumor markers in solid neoplasms (CEA, ferritin, alpha 1-antitrypsin, parathormone and calcitonin). *Boll Ist Sieroter Milan* 1982, 61, 411-422.

[17] Gao WM, Kuick R, Orchekowski RP, Misek DE *et al.* Distinctive serum protein profiles involving abundant proteins in lung cancer patients based upon antibody microarray analysis. *BMC Cancer* 2005,5, 110.

[18] Borth W. Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J* 1992, 6, 3345-3353.

[19] Briese V, Willroth PO, Brock J, Straube W. Tumor markers (alpha 2-macroglobulin, secretory immunoglobulin A, pregnancy-associated alpha 2-glycoprotein) in the serum of patients with bronchial carcinoma. *Arch Geschwulstforsch* 1984, 54, 391-398.

[20] Brecht K, Simonen M, Heim J. Upregulation of alpha globin promotes apoptotic cell death in the hematopoietic cell line FL5.12. *Apoptosis* 2005, 10, 1043-1062.

[21] Armstrong SD, Robertson DH, Cheetham SA, Hurst JL, Beynon RJ. Structural and functional differences in isoforms of mouse major urinary

proteins: a male-specific protein that preferentially binds a male pheromone. *Biochem J* 2005, 391(Pt 2), 343-350.

[22] Tamura S, Fujii J, Nakano T, Hada T, Higashino K. Urinary pseudouridine as a tumor marker in patients with small cell lung cancer. *Clin Chim Acta* 1986, 154, 125-132.

[23] Lin L, Wang Y, Bergman G, Kelloff GJ *et al.* Detection of differentially expressed genes in mouse lung adenocarcinomas. *Exp Lung Res* 2001, 27, 217-229.

[24] van Bennekum AM, Wei S, Gamble MV *et al.* Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice. *J Biol Chem* 2001, 276, 1107-1113.

[25] Roberts D, Williams SJ, Cvetkovic D *et al.* Decreased expression of retinol-binding proteins is associated with malignant transformation of the ovarian surface epithelium. *DNA Cell Biol 2002*, 21, 11-19.

[26] Zhang Z, Bast RC Jr, Yu Y, Li J *et al.* Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004, 64, 5882-5890.

[27] Moore LE, Fung ET, McGuire M, Rabkin CC *et al.* Evaluation of apolipoprotein A1 and posttranslationally modified forms of transthyretin as biomarkers for ovarian cancer detection in an independent study population. *Cancer Epidemiol Biomarkers Prev* 2006, 15, 1641-1646.

[28] Maciel CM, Junqueira M, Paschoal ME, Kawamura MT *et al.* Differential proteomic serum pattern of low molecular weight proteins expressed by adenocarcinoma lung cancer patients. *J Exp Ther Oncol* 2005, 5, 31-38.

[29] Feng JT, Liu YK, Song HY, Dai Z *et al.* Heat-shock protein 27: a potential biomarker for hepatocellular carcinoma identified by serum proteome analysis. *Proteomics* 2005, 5, 4581-4588.

[30] Goufman EI, Moshkovskii SA, Tikhonova OV, Lokhov PG *et al.* Two-dimensional electrophoretic proteome study of serum thermostable fraction from patients with various tumor conditions. *Biochemistry (Mosc)* 2006, 71, 354-360.

[31] Petrini M, Galbraith RM, Emerson DL, Nel AE, Arnaud P. Structural studies of T lymphocyte Fc receptors. Association of Gc protein with IgG binding to Fc gamma. *J Biol Chem* 1985, 260, 1804-1810.

[32] Yamamoto N, Naraparaju VR, Asbell SO. Deglycosylation of serum vitamin D3-binding protein leads to immunosuppression in cancer patients. *Cancer Res* 1996, 56, 2827-2831.

[33] Hlavaty JJ, Partin AW, Shue MJ, Mangold LA *et al.* Identification and preliminary clinical evaluation of a 50.8-kDa serum marker for prostate cancer. *Urology* 2003, 61, 1261-1265.

[34] Woodworth CD, Kreider JW, Mengel L, Miller T *et al*. Tumorigenicity of simian virus 40-hepatocyte cell lines: effect of in vitro and in vivo passage on expression of liver-specific genes and oncogenes. *Mol Cell Biol* 1988, 8, 4492-4501.

[35] Misra UK, Pizzo SV. Potentiation of signal transduction mitogenesis and cellular proliferation upon binding of receptor-recognized forms of alpha2-macroglobulin to 1-LN prostate cancer cells. *Cell Signal* 2004, 16, 487-496.

[36] Woong-Shick A, Sung-Pil P, Su-Mi B, Joon-Mo L *et al.* Identification of hemoglobin-alpha and -beta subunits as potential serum biomarkers for the diagnosis and prognosis of ovarian cancer. *Cancer Sci* 2005, 96, 197-201.

[37] Kaur G, Belotti D, Burger AM, Fisher-Nielson K *et al.* Antiangiogenic properties of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin: an orally bioavailable heat shock protein 90 modulator. *Clin Cancer Res* 2004, 10, 4813-4821.

[38] Gniadecki R. Activation of Raf-mitogen-activated protein kinase signaling pathway by 1,25-dihydroxyvitamin D3 in normal human keratinocytes. *J Invest Dermatol* 1996, 106, 1212-1217.

[39] Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol* 2006, 33, 369-385. Review.

[40] Maihle NJ, Baron AT, Barrette BA, Boardman CH *et al.* EGF/ErbB receptor family in ovarian cancer. *Cancer Treat Res* 2002, 107, 247-258. Review.

[41] Baron AT, Lafky JM, Connolly DC *et al.* A sandwich type acridinium-linked immunosorbent assay (ALISA) detects soluble ErbB1 (sErbB1) in normal human sera. *J Immunol Methods* 1998, 219, 23–43.

[42] Baron AT, Lafky JM, Boardman CH *et al.* Serum sErbB1 and epidermal growth factor levels as tumor biomarkers in women with stage III or IV epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 1999, 8, 129–137.

[43] Baron AT, Cora EM, Lafky JM *et al.* Soluble epidermal growth factor receptor (sEGFR/sErbB1) as a potential risk, screening, and diagnostic serum biomarker of epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2003, 12, 103–113.

[44] Schwartz EE, Winsten S. Serum properdin in tumor-bearing mice. I. Comparison with natural and immune antibodies. *Cancer Res* 1964, 24, 825-829.

[45] Schwartz EE, Winsten S. Serum properdin in tumor-bearing mice. II. The influence of tumors of different origin. *Cancer Res* 1964, 24, 830-834.

Figure 1

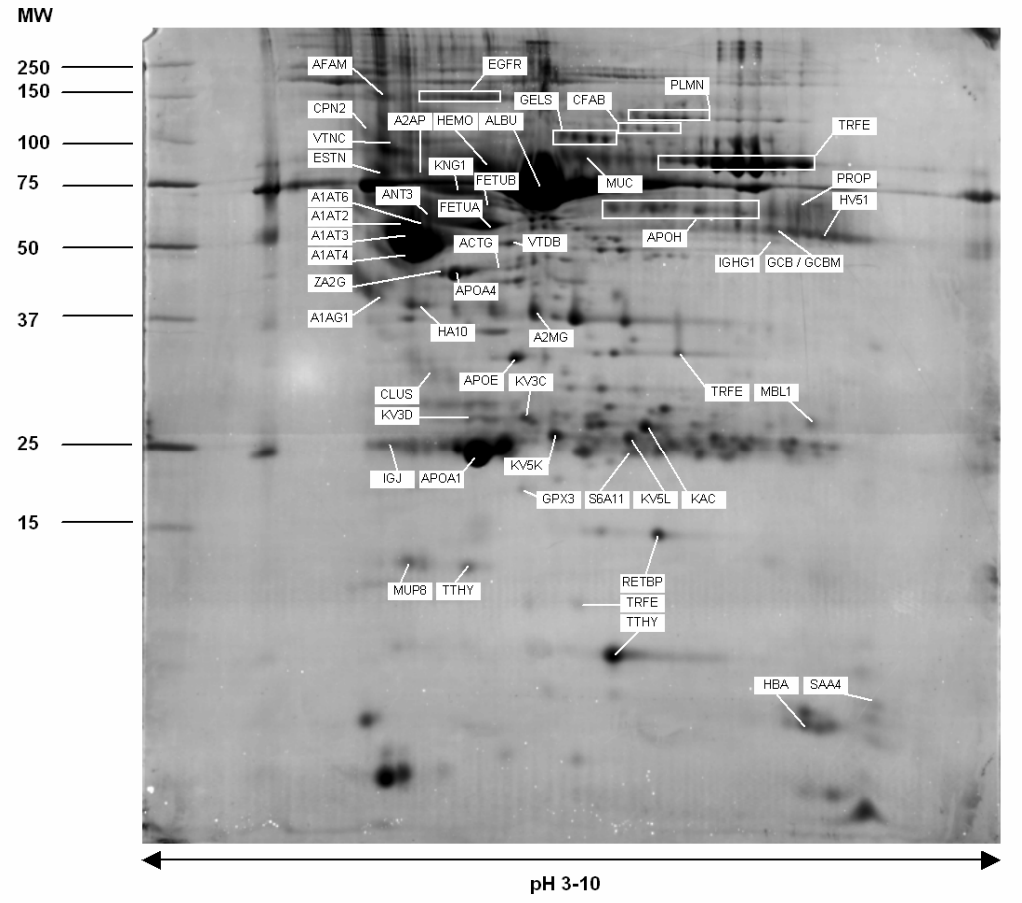

MW
250
150
100
75
50
37
25
15
AFAM
EGFR
PLMN
GELS
CFAB
CPN2
A2AP
HEMO
ALBU
TRFE
VTNC
ESTN
KNG1
FETUB
MUC
PROP
HV51
A1AT6
ANT3
FETUA
A1AT2
A1AT3
ACTG
VTDB
APOH
A1AT4
IGHG1
GCB / GCBM
ZA2G
APOA4
A1AG1
HA10
A2MG
APOE
KV3C
TRFE
MBL1
CLUS
KV3D
KV5K
IGJ
APOA1
GPX3
S6A11
KV5L
KAC
MUP8
TTHY
RETBP
TRFE
TTHY
HBA
SAA4
pH 3-10

Figure 2

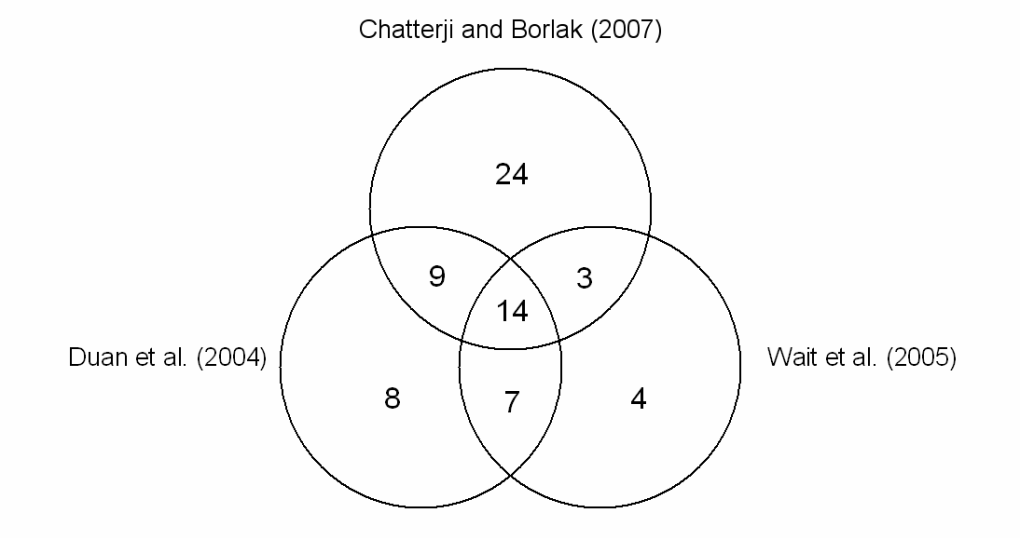

Chatterji and Borlak (2007)
24
9
3
14
Duan et al. (2004)
Wait et al. (2005)
8
7
4

Figure 3a

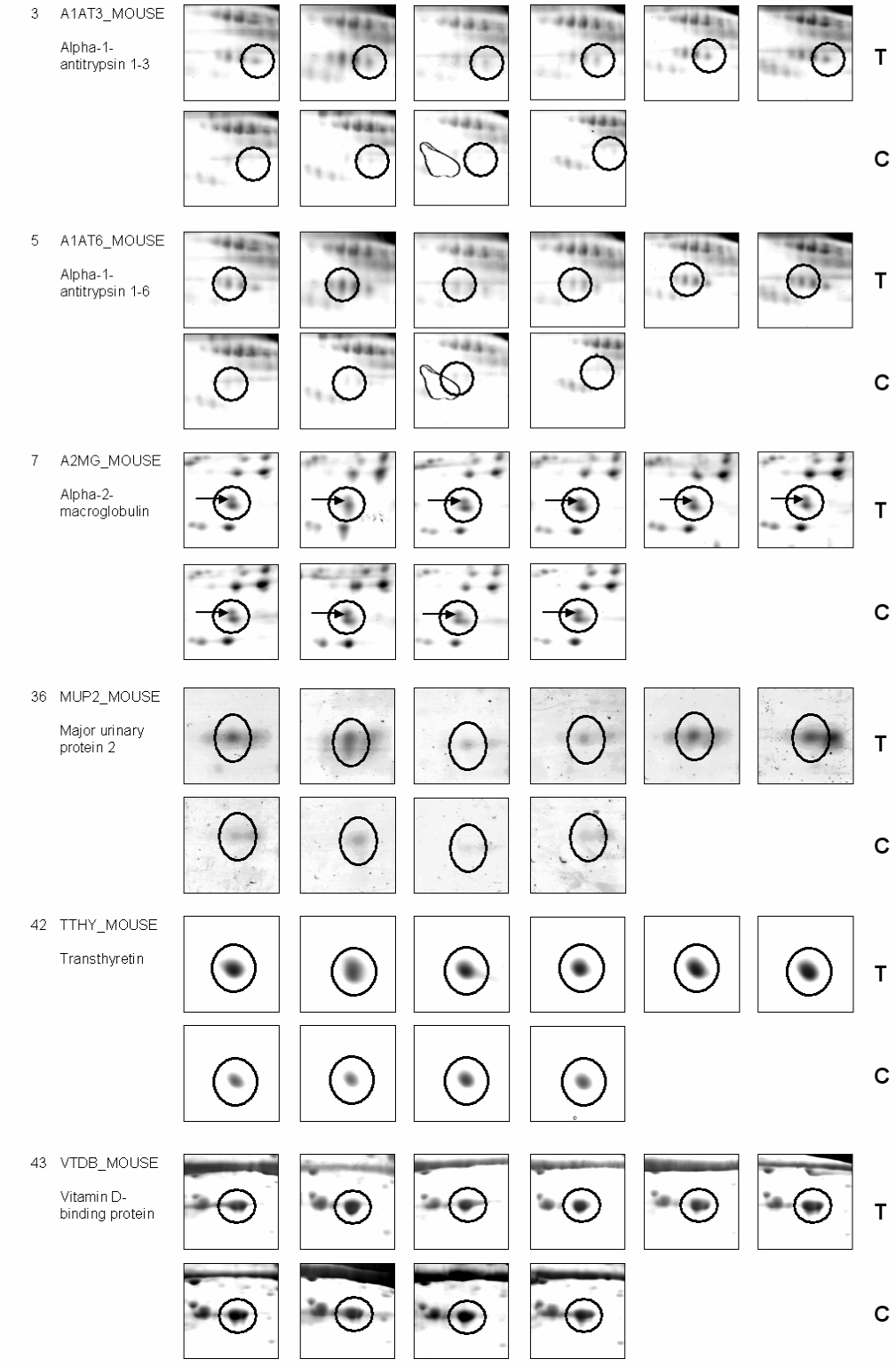

3 A1AT3_MOUSE
Alpha-1-antitrypsin 1-3
T
C
5 A1AT6_MOUSE
Alpha-1-antitrypsin 1-6
T
C
7 A2MG_MOUSE
Alpha-2-macroglobulin
T
C
36 MUP2_MOUSE
Major urinary protein 2
T
C
42 TTHY_MOUSE
Transthyretin
T
C
43 VTDB_MOUSE
Vitamin D-binding protein
T
C

Figure 3b

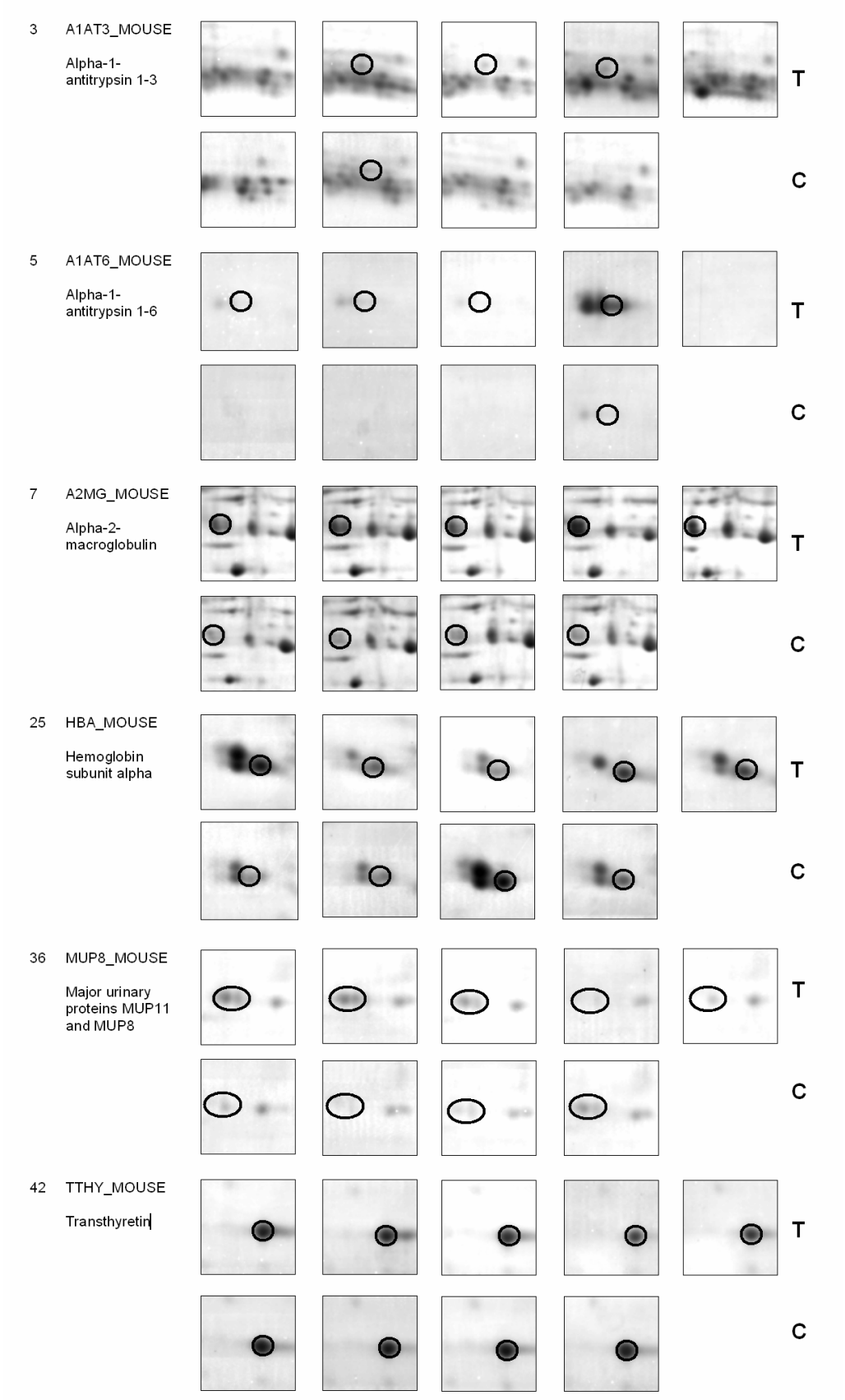

3
A1AT3_MOUSE
Alpha-1-antitrypsin 1-3
T
C
5
A1AT6_MOUSE
Alpha-1-antitrypsin 1-6
T
C
7
A2MG_MOUSE
Alpha-2-macroglobulin
T
C
25
HBA_MOUSE
Hemoglobin subunit alpha
T
C
36
MUP8_MOUSE
Major urinary proteins MUP11 and MUP8
T
C
42
TTHY_MOUSE
Transthyretin
T
C

Figure 4

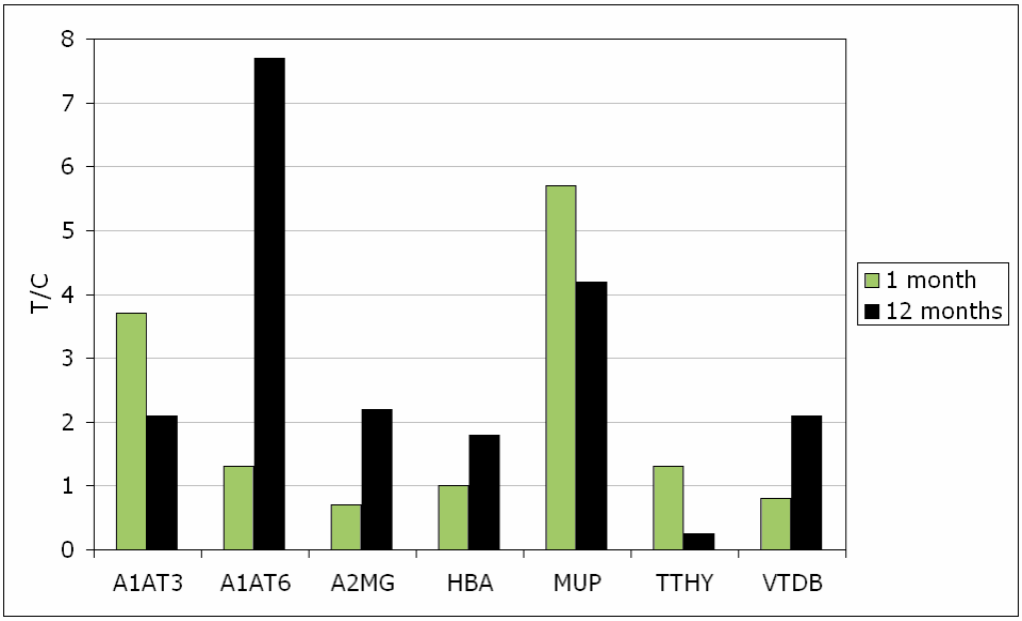

8
7
6
5
4
3
2
1
0
T/C
A1AT3
A1AT6
A2MG
HBA
MUP
TTHY
VTDB
1 month
12 months

Figure 5

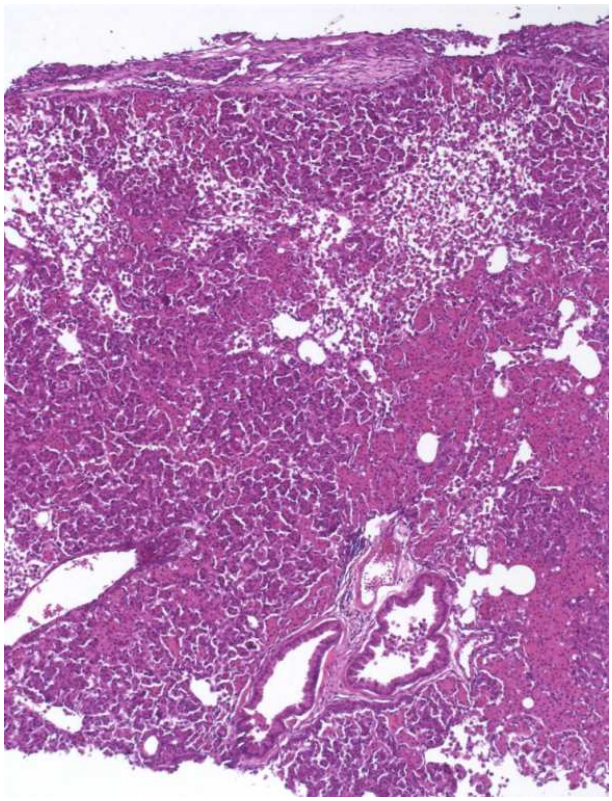


Table 1

No.	Protein identification		Protein superfamily
1	A1AG1_MOUSE	Alpha-1-acid glycoprotein 1 (Orosomucoid-1)	Lipocalin
2	A1AT2_MOUSE	Alpha-1-antitrypsin 1-2	Serpin
3	A1AT3_MOUSE	Alpha-1-antitrypsin 1-3	Serpin
4	A1AT4_MOUSE	Alpha-1-antitrypsin 1-4	Serpin
5	A1AT6_MOUSE	Alpha-1-antitrypsin 1-6	Serpin
6	A2AP_MOUSE	Alpha-2-antiplasmin	Serpin
7	A2MG_MOUSE	Alpha-2-macroglobulin	Protease inhibitor I39
8	ACTG_MOUSE	Actin, cytoplasmic 2 Gamma-actin	Actin
9	AFAM_MOUSE	Afamin (Alpha-albumin)	ALB/AFP/VDB
10	ALBU_MOUSE	Serum albumin	ALB/AFP/VDB
11	APOA1_MOUSE	Apolipoprotein A-I	Apolipoprotein A1/A4/E
12	APOA4_MOUSE	Apolipoprotein A-IV	Apolipoprotein A1/A4/E
13	APOE_MOUSE	Apolipoprotein E	Apolipoprotein A1/A4/E
14	APOH_MOUSE	Apolipoprotein H (Beta-2-glycoprotein 1)	Complement control module/SCR domain
15	CFAB_MOUSE	Complement factor B (C3/C5 convertase)	Peptidase S1
16	CLUS_MOUSE	Apolipoprotein J (Clusterin)	Clusterin
17	CPN2_MOUSE	Carboxypeptidase N subunit 2	
18	ESTN_MOUSE	Liver carboxylesterase N (Lung surfactant convertase)	Type-B carboxylesterase/ lipase
19	FETUA_MOUSE	Fetuin A (Alpha-2-HS-glycoprotein)	Fetuin
20	FETUB_MOUSE	Fetuin B	Fetuin
21	GCB_MOUSE	Ig gamma-2B chain C region secreted form	Immunglobulin
22	GELS_MOUSE	Gelsolin (Brevin)	Villin/gelsolin
23	GPX3_MOUSE	Glutathione peroxidase 3	Glutathione peroxidase
24	HA10_MOUSE	H-2 class I histocompatibility antigen, Q10 alpha chain	MHC class 1
25	HBA_MOUSE	Hemoglobin subunit alpha (Alpha-globin)	Globin
26	HEMO_MOUSE	Hemopexin	Hemopexin-like domain
27	HV51_MOUSE	Ig heavy chain V region AC38 205.12	Immunglobulin
28	IGHG1_MOUSE	Ig gamma-1 chain C region secreted form	Immunglobulin
29	KAC_MOUSE	Ig kappa chain C region	Immunglobulin
30	KNG1_MOUSE	Kininogen-1	
31	KV3D_MOUSE	Ig kappa chain V-III region 50S10.1	Immunglobulin
32	KV5K_MOUSE	Ig kappa chain V-V region HP R16.7	Immunglobulin
33	KV5L_MOUSE	Ig kappa chain V-V region HP 93G7	Immunglobulin
34	MBL1_MOUSE	Mannose-binding protein A	C-type lectin like
35	MUC_MOUSE	Ig mu chain C region secreted form	Immunglobulin
36	MUP8_MOUSE	Major urinary proteins 11 and 8 (MUP8)	Lipocalin
37	PLMN_MOUSE	Plasminogen	plasminogen/peptidase S1
38	RETBP_MOUSE	Plasma retinol-binding protein	Lipocalin
39	S6A11_MOUSE	Sodium- and chloride-dependent GABA transporter 4	SNF (sodium neurotransmitter symporter)
40	SAA4_MOUSE	Serum amyloid A-4 protein	SAA
41	TRFE_MOUSE	Serotransferrin	Transferrin
42	TTHY_MOUSE	Transthyretin (Prealbumin)	Transthyretin
43	VTDB_MOUSE	Vitamin D-binding protein (Gc-globulin)	ALB/AFP/VDB
44	VTNC_MOUSE	Vitronectin	Somatomedin B domain
45	ZA2G_MOUSE	Zinc-alpha-2-glycoprotein	MHC class 1

Table 2a

No.	Protein ID	Tumor (T1)	Tumor (T2)	Tumor (T3)	Tumor (T4)	Tumor (T5)	Tumor (T6)	Control (C1)	Control (C2)	Control (C3)	Control (C4)	Ratio (T/C)
3	A1AT3_MOUSE	3825	3948	3698	3641	2769	2488	1562	643	281	1211	**3.7**
5	A1AT6_MOUSE	2477	4772	1325	2462	2742	2644	1700	1240	2067	3606	**1.3**
7	A2MG_MOUSE	2683	319	3362	3404	1452	1345	2253	5181	1723	3635	**0.7**
25	HBA_MOUSE	39568	18081	4104	27938	24936	20095	32241	12354	21703	21248	**1.0**
36	MUP2_MOUSE	1499	1240	47	309	1933	1326	327	300	77	41	**5.7**
42	TTHY_MOUSE	36920	27522	28645	33670	34577	30648	19699	23241	28600	26460	**1.3**
43	VTDB_MOUSE	12981	19652	22967	24802	19831	16896	21342	25219	26614	29599	**0.8**

Table 2b

No.	Protein ID	Tumor (T1)	Tumor (T2)	Tumor (T3)	Tumor (T4)	Tumor (T5)	Control (C1)	Control (C2)	Control (C3)	Control (C4)	Ratio (T/C)
3	A1AT3_MOUSE	-	538	234	186	-	-	152	-	-	**2.1**
5	A1AT6_MOUSE	100	68	6	654	-	-	-	-	27	**7.67**
7	A2MG_MOUSE	506	1124	610	872	655	536	219	155	469	**2.18**
25	HBA_MOUSE	4990	2161	627	3176	3938	479	365	3608	2228	**1.79**
29	KAC_MOUSE	1146 375 461 478	897 281 1465 1509	- 393 1972 1482	566 635 1365 2660	1420 786 1176 2911	214 347 1009 2194	176 51 138 247	703 297 244 167	751 104 223 144	**2.15** **2.47** **3.19** **2.63**
36	MUP8_MOUSE	2198 1134	2333 786	1882 298	34 111	93 481	114 176	25 57	136 62	1852 236	**2.46** **4.23**
39	S6A11_MOUSE	203 600	154 1763	56 2439	742 1095	503 5878	- 1294	- 103	- 649	- 206	**T** **4.18**
42	TTHY_MOUSE	111	479	84	21	50	276	417	1324	335	**0.25**
43	VTDB_MOUSE	3096	2466	4026	459	4149	2375	1124	1088	878	**2.08**
46	EGFR_MOUSE (Epidermal growth factor receptor)	-	-	-	-	-	365	695	846	232	**C**
47	GCBM_MOUSE (Ig gamma-2B chain C region, membrane-bound form)	-	-	-	-	-	1548	1904	302	1509	**C**
48	IGJ_MOUSE (Ig J chain)	1156	1428	1853	766	5384	-	-	-	-	**T**
49	KV3C_MOUSE (Ig kappa chain V-III region MOPC 70)	478	1509	1482	2660	2911	-	-	-	-	**T**
50	PROP_MOUSE (Properdin)	-	-	-	-	-	400	509	887	334	**C**

Supplementary Table 1:
A summary of serum proteins from SP-C/c-raf transgenic mice

No. (internal no.)	Swissprot ID	Swissprot Accession no.	Protein aliases	Mascot score No. of matched peptides Percentual coverage	Peptide sequences	Gene	Subcellular location	Protein function / notes
1 (418)	A1AG1_MOUSE	Q63593	Alpha-1-acid glycoprotein 1 [Precursor] AGP 1 Orosomucoid-1 OMD 1	84.5 7 85.7	AVTHVGMDESEIFVDWKK + OXPM HGAFMLAFDLK.DEK HGAFMLAFDLK.DEK + OXPM RPDITPELR RPDITPELREVFQK YEGGYETFAHLVLR YEGGYETFAHLVLRK	Orm1 Agp1		Appears to function in modulating the activity of the immune system during the acute-phase reaction.
2 (1621)	A1AT2_MOUSE	P22599	Alpha-1-antitrypsin 1-2 [Precursor] Serine protease inhibitor 1-2 Alpha-1 protease inhibitor 2 Alpha-1-antiproteinase AAT	183.3 24 46.5	AVHKAVLTIDETGTEAAAATVTEAVPMSMPPILR AVLTIDETGTEAAAATVTEAVPMSMPPILR AVLTIDETGTEAAAATVTEAVPMSMPPILR + 2 OXPM AVLTIDETGTEAAAATVTEAVPMSMPPILR + OXPM DQSPASHEIATNLGDFAISLYR FDHPFLFIFEEHTQSPIFVGK FLEEAKNHYQAEVFSVNFAESEEAK IFNNIGADLSGITEENAPLK IFNNIGADLSGITEENAPLK.LSK IYEAVKELDQDTVFALAIYLEFK KPFDPENTEEAEFHVDK KPFDPENTEEAEFHVDKSTTVK LSISGDYNLK LSISGDYNLKTLMSPLGTR LSISGDYNLKTLMSPLGTR + OXPM LVQIHPR LVQIHPRLSISGDYNLK MQHLEQTLNKELSK RLVQIHPR SFQHLLQTLNRPDSELQLST GNGLFV NNDLK TLMSPLGTFR TLMSPLGTFR + OXPM VINDFVEK.GTQGK VKKPFDPENTEEAEFHVDK	Spi1-2 Aat2 Dom2	Secreted	Inhibitor of serine proteases. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin.
3 (2632)	A1AT3_MOUSE	Q00896	Alpha-1-antitrypsin 1-3 [Precursor] Serine protease inhibitor 1-3 Alpha-1 protease inhibitor 3	223.0 23 73.3	AVLTIDETGTEAAAATVLLAVPYSMPPIVR + 2 OXPM AVLTIDETGTEAAAATVLLAVPYSMPPIVR + OXPM DQSPASHEIATNLGDFAISLYR FDHPFLFIFEEHTQSPLFVGK FLEEAKNHYQAEVFSVNFAESEEAK FLLKRPR IFNNIGADLSGITEENAPLK IFNNIGADLSGITEENAPLKLSQAVHK KLDQDTVFALANYLEFK KPFDPENTEEAEFHVDESTTVK LAQHFPR LSISGEYNLK MQHLEQTLSK NHYQAEVFSVNFAESEEAK NHYQAEVFSVNFAESEEAKK RLAQHFPR SFQHLLQTLNRPDSELQLST GNGLFV NNDLK SFQHLLQTLNRPDSELQLST GNGLFV NNDLKLVEK TLMSPLGTFR TLMSPLGTFR + OXPM TLMSPLGTFRFNNIGADLSGITEENAPLK + OXPM VINDFVEK VINDFVEK.GTQGK VKQPFDPENTEEAEFHVDESTTVK	Spi1-3 Dom3	Secreted	Inhibitor of serine proteases. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin.
4 (1613)	A1AT4_MOUSE	Q00897	Alpha-1-antitrypsin 1-4 [Precursor] Serine protease inhibitor 1-4 Alpha-1 protease inhibitor 4	233.0 23 53.3	AVLTIDETGTEAAAATVLQVATYSMPPIVR + OXPM DQSPASHEIATNLGDFALR ELISQFLLNR ELISQFLLNRR FDHPFLFIFEEHTQSPIFVGK IFNNIGADLSGITEENAPLK IFNNIGADLSGITEENAPLK.LSK KLDQDTVFALANYLEFK LDQDTVFALANYLEFK LSISGNYNLK NHYQAEVFSVNFAESEEAK NHYQAEVFSVNFAESEEAKK QPFDPENTEEAEFHVDESTTVK RSDAQIHPR SDAQIHPR SFQHLLQTLNRPDSELQLST GNGLFV NNDLK SFQHLLQTLNRPDSELQLST GNGLFV NNDLKLVEK TLMSPLGTFR TLMSPLGTFR + OXPM TLMSPLGTFRFNNIGADLSGITEENAPLK + OXPM VINDFVEK VINDFVEKGTQGK VKQPFDPENTEEAEFHVDESTTVK	Spi1-4 Dom4	Secreted	Inhibitor of serine proteases. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin.
5 (1612)	A1AT6_MOUSE	P81135	Alpha-1-antitrypsin 1-6 [Precursor] Serine protease inhibitor 1-6 Alpha-1 protease inhibitor 6	145.3 12 78.4	DQSPASHEIATNLGDFAISLYR ELISKFLLNR FDHPFLFIFEEHTQSPLFVGK IFNNIGADLSGITEENAPLK KLDQDTVFALANYLEFK LAQHFPR NHYQAEVFSVNFAESEEAKK RLAQHFPR SFQHLLQTLNRPDSELQLST GNGLFV NNDLK TLMSPLGTFR TLMSPLGTFR + OXPM TLMSPLGTFRFNNIGADLSGITEENAPLK + OXPM	Spi1-6 Dom6	Secreted	Inhibitor of serine proteases. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin.
6 (2633)	A2AP_MOUSE	Q61247	Alpha-2-antiplasmin [Precursor] Alpha-2-plasmin inhibitor Alpha-2-PI Alpha-2-AP	93.4 8 63.3	GFPIKDDFLEQSER LAPNEEDFYFSSPK + OXPM LDHQDFGDHATLK LDHQDFGDHATLKR LIQQHIDKADFANIGGK NPNPSALPQLQEQR SVPTAEETRR TFGPDLKLAPR	Pi	Secreted	The major targets of this inhibitor are plasmin and trypsin, but it also inactivates chymotrypsin.
7 (4853)	A2MG_MOUSE	Q61838	Alpha-2-macroglobulin [Precursor] Alpha-2-M Pregnancy zone protein	133.3 12 77.3	AAPLSLCALTAVDQSVLLLKPEAK ABYLISGYQR ALLAYAFALAGNK ALSFYQPR EEGTGIELTGKGSCEIANALSKLK HSLGDNDAHSIFQSVGNIFTNSK KTVSAVATPK LSPDSYNLLPGK LTEVPALVHKDTVVK SKAIHYLSGYQR VKALSFYQPR VNITNYRPGLPFSGQVLLVDEK	A2m Pzp	Secreted	Is able to inhibit all four classes of proteinases by a unique 'trapping' mechanism. This protein has a peptide stretch, called the 'bait region' which contains specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced in the protein which traps the proteinase. The entrapped enzyme remains active against low molecular weight substrates (activity against high molecular weight substrates is greatly reduced). Following cleavage in the bait region a thioester bond is hydrolyzed and mediates the covalent binding of the protein to the proteinase.
8 (4533)	ACTG_MOUSE	P63713	Actin, cytoplasmic 2 Gamma-actin	94.9 6 97.7	AVFPSIVGRPR GYSFTTTAEREIVR LDLAGRDLTDYLMK + OXPM QEYDESGPSIVHR SVELPDGQVITIGNER VAPEEHPVLLTEAPLNPK	Actg1 Actg	Cytoplasma	Cell structure and mobility proteins: actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
9 (2838)	AFAM_MOUSE	O89023	Afamin [Precursor] Alpha-albumin Alpha-Alb	189.3 22 53.8	AAPEQYLK AAPQLPMEELVSLSK AAPQLPMEELVSLSK + OXPM AHVGFLPPFPTLDPEEK CVADHTLPECSK DSDPDKFFAEFIVEYSR EACFMANIKDDRPEGLSLR EAKFTESEIHVCQER EDLQNKK ENPAGCYR FTESEIHVCQER HCFEHLK HFLVKFTK RLCFFYNK TDFAFRR TINPAVDHCCK TINPAVDHCCKTDFAFR VLNSRNVACFSK VMLDYRDR VMLDYRDR + OXPM VYMDFLEDCCSR VYMDFLEDCCSR + OXPM	Afm	Secreted	Possible role in the transport of yet unknown ligand.

13 (1732)	ALBU_MOUSE	P07724	Serum albumin [Precursor]	455.0 39 95.7	AADKDTCFSTEGPNLVTR AETFTFHSDICTLPEKEK AFKAWAVAR APQVSTPTLVEAAR CCAEANPPACYGTVLAEFQPLVEEPK CCSQSLVER CCTLPEDQRLPCVEDYLSAILNR CSYDEHAK CSYDEHAKLVQEVTDFAK DVFLGTFLYEYSR EAHKSEIAHR EHPTTFMGHYLHEVAR EHPTTFMGHYLHEVAR + OXPM HPDYSVSLLLR KYEATLEK LCAIPHLR LCAIPHLRENYGELADCCTK LGEYGFQNALIVR LPCVEDYLSAILNR LQTCCDKPLLK LQTCCDKPLLKK LSQTFPNADFAEITK LSQTFPNADFAEITKLATDLTK LVQEVTDFAK QTALAELVK RHPDYSVSLLLR RPCFSALTVDETYVPK RPCFSALTVDETYVPKEFK SLHTLFGDKLCAIPHLR TNCDLVEKLGEYGFQNALIVR TPVSEHVTK TVMDDFAQFLDTCCK TVMDDFAQFLDTCCK + OXPM VCLLHEK VCLLHEKTPVSEHVTK YNAETEKQATESK + OXPM YNDLQEQHFK YTQKAPQVSTPTLVEAAR	Alb Alb1 Alb-1	Secreted	Serum albumin, the main protein of plasma, has a good binding capacity for water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood.
11 (2312)	APOA1_MOUSE	Q03623	Apolipoprotein A-I [Precursor] Apo-AI ApoA-I	238.0 19 87.3	ARPALEDLR DFANVYVDAVKDSGR DFNVDNLEK DFNVDNLEKETDWVR HSLMPMLETLK LAELKSNPTLNEYHTR LSPVAEEFR LSPVAEEFRDR QKLQELQGR SNPTLNEYHTR TQLAPHSEQMR TQLAPHSEQMR + OXPM TQLAPHSEQMRESLAQR + OXPM VAPLGAELQESAR VAPLGAELQESARQK VKDFANVYVDAVK VQPYLDEFQK VQPYLDEFQKK VWKEDVELYR	Apoa1	Secreted	APOA1 participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase.
12 (2311)	APOA4_MOUSE	P06728	Apolipoprotein A-IV [Precursor] Apo-AIV ApoA-IV	296.0 24 83.4	ALVQQLEQFR ALVQQLEQFRQQLGPHSGEVESHLSFLEK ATEQVILEDLRR EAVEQFQKTDVTQQLSTLFQDK EKVNEFMSTLEK + OXPM KGSPDQPQALPLPGAQEQAQEQAQEQVQPKPLES LGDASTYADGVHNK LNHQMEGLAFQMKK + 2 OXPM LNHQMEGLAFQMKK + 2 OXPM LQEHLKPYAVDLQDQINTQTQEMK + OXPM LQLTPVQR LVPFVVQLSGHLAKETER MMPMANKVTQFFGENMQK + 3 OXPM NLALVEDVQSK NMEELKGHLTPR + OXPM QLEQQVEEFRR QQLGPHSGEVESHLSFLEK SLALTVGVQEK TDVTQQLSTLFQDKLGDASTYADGVHNK TVEPMQEMLTPK + 2 OXPM VKGNTEGLQK VNEFMSTLEK + OXPM VNEFMSTLEKK + OXPM VSAKIDQLQK	Apoa4	Secreted	May have a role in chylomicrons and VLDL secretion and catabolism. Required for efficient activation of lipoprotein lipase by ApoC-II; potent activator of LCAT. Apoa-IV is a major component of HDL and chylomicrons.

13 (3429)	APOE_MOUSE	P38226	Apolipoprotein E [Precursor] Apo-E	198.0 15 95.3	AQAFQDRIR ELEEQLQPVAEETR ELEEQLQPVAEETRAR FWDYLR GRLEEYQKHQAR GVSAIRER LGADMEDLRNR + OXPM LGKEYQAAQAAR LGPLVEQGR LGPLVEQGRQR LKGWEPVFEDMHR + OXPM LQAEFQAR QWAAILMEK SKMEEQTQQIR + OXPM TANLGAGAAQPLRDR	Apoe	Secreted	Mediates the binding, internalization, and catabolism of lipoprotein particles. It can serve as a ligand for the LDL (apo B/E) receptor and for the specific apo-E receptor (chylomicron remnant) of hepatic tissues.
14 (6736)	APOH_MOUSE	Q31339	Beta-2-glycoprotein 1 [Precursor] Beta-2-glycoprotein I Apolipoprotein H Apo-H B2GPI Beta(2)GPI Activated protein C-binding protein APC inhibitor	223.0 23 64.5	ATFGCHETYKLDGPEEAECTK ATVLYQGMR ATVLYQGMR + OXPM CPFPPRPENGYVNYPAKPVLLYK CPFPPRPENGYVNYPAKPVLLYKDK CSYTVEAHCR CTEEGKVVSPDIPACAR DGTIEIPSCFK DGTEIEIPSCFKEHSSLAFWK EHSSLAFWK FTCPLTGMVPFNTLR FTCPLTGMVPFNTLR + OXPM ICPKPDDLPFATVVVPLK IFCPPPVPK KATVLYQGMR + OXPM KCSYTVEAHCR LPECLEVK NGMVHHQDKIHFYCK + 2 OXPM TGTWSFLPTCR TSYDPGEQIVYSCKPGYVSR VCPFAGILENGIVR VKLQEQFK WSPDIPACAR	Apoh B2gp1	Secreted	Binds to various kinds of negatively charged substances such as heparin, phospholipids, and dextran sulfate. May prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells.
15 (6832)	CFAB_MOUSE	P34186	Complement factor B [Precursor] EC 3.4.21.47 C3/C5 convertase	181.0 18 71.0	ALLDIGRDPK ALRJPQTATSEAK CLTNLEKVASYGVRPCR CPRPQDFEINGEFVKPR DLEIEEVLFHPK GNDYHKQPWQAK KAEGPEFYDYDVALVFK KDNEHHVFK LEDVTYHCSR QQLVPSYAR RDLEEEVLFHPK RQQLVPSYAR STGSWSDLQTR VASYGVRPR VGSQVRLEDNTYHCSR VKDASEVVTPR YGGTLRPICLPCTEGTTR	Cfb Bf H2-Bf	Secreted	Factor B which is part of the alternate pathway of the complement system is cleaved by factor D into 2 fragments: Ba and Bb. Bb, a serine protease, then combines with complement factor 3b to generate the C3 or C5 convertase.
16 (1138)	CLUS_MOUSE	Q06890	Clusterin [Precursor] Sulfated glycoprotein 2 SGP-2 Clustrin Apolipoprotein J Apo-J	73.5 7 43.0	ASGIDTLFQDR ASGIDTLFQDRFFAR ELHDPMVFSPRDFPHRPHFLYPK HTCMKFYAR + OXPM QQSQVLDAMQDSFAR + OXPM RPHFLYPK YINKEIQNAVQGVK	Clu Apoj Mfgp-2	Secreted	Not yet clear. It is known to be expressed in a variety of tissues and it seems to be able to bind to cells, membranes and hydrophobic proteins. It has been associated with programmed cell death.
17 (1813)	CPN2_MOUSE	Q9DBB9	Carboxypeptidase N subunit 2 [Precursor] Carboxypeptidase N polypeptide 2 Carboxypeptidase N 83 kDa chain Carboxypeptidase N regulatory subunit Carboxypeptidase N large subunit	137.0 12 62.7	AAHSQCAVSNPEGTVLACEESR ALGLDEGEPAGSWDLTVEGR CRVLNRQLSSR GAFQSLTGLQMLK + OXPM GQLVPHLQEQLQCPVNPGHLSFR HLEPDAFGGLPR LQLLNLSR LSNNMLAR + OXPM TLNLAQNLLTQLPK TLPGRLFQSLR VVFLHTQVR WLNRQLSSR	Cpn2	Secreted	The B3 kDa subunit binds and stabilizes the catalytic subunit at 37 degrees Celsius and keeps it in circulation. Under some circumstances it may be an allosteric modifier of the catalytic subunit.

18 (1739)	EST N_MOUSE	P23953	Liver carboxylesterase N [Precursor] EC 3.1.1.1 PES-N Lung surfactant convertase	146.0 2.0 73.1	AISESGVYIHFTNVGKK APEELAEK DAGFSTYMYEFR DAGFSTYMYEFR + OXIM EILPLKISEDCLYLNIYSPADLTK FAPPQPAEPWSFVK FVVANIFAR IGEDCLYLNIYSPADLTK MPEETASLLLR MPEETASLLLR + OXIM MPEETASLLLRR MPEETASLLLRR + OXIM MPVMPVVANIFAR + OXIM NGNPNGEGLPHWPEYDEKEGYLQIGATTQQAQR QKTESELLEISGK SLRDAGFSTYMYEFR SLRDAGFSTYMYEFR + OXIM VLGKYISLEGFEQPVAVFLGFPFAKPPLGSLR YISLEGFEQPVAVFLGFPFAKPPLGSLR YRPSFVSDK	Es1	Endo-plasmatic reticulum	Involved in the detoxification of xenobiotics and in the activation of ester and amide prodrugs. Involved in the extracellular metabolism of lung surfactant.
19 (2714)	FETUA_MOUSE	P29699	Alpha-2-HS-glycoprotein [Precursor] Fetuin-A Countertrypin	133.0 1.0 39.4	AHLMHNLGGEEVSVACK AHLMHNLGGEEVSVACK + OXIM AQFVFLPVSTLVEFVIAATDCTAK AQFVFLPVSTLVEFVIAATDCTAKEVTDPAK GLSDHRTVHDLR HAFSPVASVESASGETLHSPK QLTEHAVEGDCDFHILK QLTEHAVEGDCDFHILKQDGQFR VGQPQAAGPVSPMCPGR VGQPQAAGPVSPMCPGR + OXIM	Ahsg Fetua	Secreted	Probably involved in differentiation.
23 (3737)	FETUB_MOUSE	Q9QXC1	Fetuin-B [Precursor] IRL685	114.0 1.0 74.5	AMFHNIKPR AMFHNIKPR + OXIM AMFHNIKPRR + OXIM DGYMLSLNR + OXIM DQKDGYMLSLNR + OXIM HVPLQPVEK LVVLPPPGKEQR MFYESVYGQCK + OXIM SAECPGPEKENNPLVLPP SQASCSLQMGDSEPVGECQGSTVQSSLR	Fetub	Secreted	
21 (7632)	GCB_MOUSE	P31866	Ig gamma-2B chain C region secreted form	116.0 1.2 42.0	APQVYLPPPAEQLSR APQVYLPPPAEQLSRK DLPSPIER GLVRAPQVYLPPPAEQLSR HEGLKNYYLK LEPSGPRSTIPPCPPCK LEPSGPRSTIPPCPPCKECHK TDSPSCNVFR TDSPSCNVFRHEGLK TTPPSVYPLAPGCGDTTGSSVTLGCLVK VNHKDLPSPIER WEKTDSPSCNVFR		Secreted	
22 (4825)	GELS_MOUSE	P13020	Gelsolin [Precursor] Actin-depolymerizing factor ADF Brevin	236.0 1.9 91.5	AGKEPGLQIWR AQERAPQSR DGDQTAPASIR EPGLQIWR EVQGFESSTFSGYFK GGVASGFKHVVPNEVVVQR HVVPNEVVVQR QTQVSVLPEGGETPLFK RTPITVFR SEDCFILDHGR SEDCFILDHGRDGK SGALNSNDAFVLK SQHVQVEEGEPDAFWEALGGK TPSAAYLWVGAGAEEADK VSEARPSTMVVEHPEFLK VSEARPSTMVVEHPEFLK + OXIM VSNGAGSMSVSLVADENPFAQGALR + OXIM YIETDPANR YIETDPANRDR	Gsn Gsb	Secreted (Isoform 1) Cytoplasma (Isoform 2)	Calcium-regulated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange (end-blocking or capping). It can promote the assembly of monomers into filaments (nucleation) as well as sever filaments already formed.
23 (3139)	GPX3_MOUSE	P46412	Glutathione peroxidase 3 [Precursor] EC 1.11.1.9 GSHPx-3 GPx-3 Plasma glutathione peroxidase GSHPx-P	76.5 5 57.7	FLVGPDGIPVMR + OXIM MDILSVMRR + 2 OXIM NSCPPTAELGSGPGR TTVSNVKMDLSVMR + 2 OXIM YVRPGGGIFVPNFQLTEK	Gpx3	Secreted	Protects cells and enzymes from oxidative damage, by catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide, by glutathione.

24 (2533)	HA13_MOUSE	P01898	H-2 class I histocompatibility antigen, Q13 alpha chain [Precursor]	273.0 1.9 93.6	AKGHEQSFHVSLR APWMEQEGPEYWER + OXIM APWMEQEGPEYWERETQR + OXIM ATLEAECYBVGLR FDSDAETPRMEPR + OXIM FIVGYVDDTQFVR FIVGYVDDTQFVRFDSDAETPR GHEQSFHVSLR GYLQYAYDGR GYLQYAYDGRDYIALNEDLK KWEQAGAAEYYR TDPPKTHVTHHPISDHEGDVTLR THVTHHPGSEGDVTLR TWTAADVAAIITR VDSDGRFLR WASVVVPLGK WEQAGAAEYYR YRTSVSRPGLGEPR YLELGKETLLR	H2-Q13	Membrane	Involved in the presentation of foreign antigens to the immune system.
25 (7311)	HBA_MOUSE	P01942	Hemoglobin subunit alpha Hemoglobin alpha chain Alpha-globin	116.0 1.0 76.0	AAWGKVGGHGAEYGAEALER IGGHGAEYGAEALER IGGHGAEYGAEALERMFASFPTTK IGGHGAEYGAEALERMFASFPTTK + OXIM LRVDPVNFK MFASFPTTK MFASFPTTK + OXIM MFASFPTTKTYFPHFDVSHGSAQVK + OXIM TYFPHFDVSHGSAQVK TYFPHFDVSHGSAQVKGHGK	Hba Hba-a1	Extracellular	Involved in oxygen transport from the lung to the various peripheral tissues.
26 (2722)	HEMO_MOUSE	Q91X72	Hemopexin [Precursor]	225.0 2.2 76.7	CSPDPGLTALLSDHR DYFVSCPGR DYFVSCPGRGHGRPR ELGSPDGLETDQAAFSCPGSSR FNPVTGEVPPR FNPVTGEVPPRYPLDAR GATYAFTGDHYWR GECQSEGVLFFQGNRK GGNNLVSGYPK GPDSYFLRLDLK KVFWDFATR LFQEEPGIPYPPDAAVECHR LWWLDLK LYVSSGRR RLVWLDLK SGAQATWTEVSWPHEKVDGALCLDK SLPQPQKVFISILGCSQ VWVYPPEVK VFFWDFATR WKNPTSVDAAFR YYCFQGNK YYCFQGNKFLR	Hpx Hpxn	Secreted	Binds heme and transports it to the liver for breakdown and iron recovery, after which the free hemopexin returns to the circulation.
27 (7614)	HVS1_MOUSE	P36333	Ig heavy chain V region AC38.205.12	56.3 4 17.4	ASGYTFTDYYMNWVK EVQLQQSGPELVKPGASVK SLEWIGDINPNNGGTSYNQK SLTSEDSAVYYCAR			
28 (7555)	IGHG1_MOUSE	P01868	Ig gamma-1 chain C region secreted form	79.8 6 42.7	APQVYTIPPKEQMAK + OXIM CPVTSAAPAPEK SNWEAGNTFTCSVLHEGLHNHHTEK SVSELPIMHQDWLHGKEFK + OXIM TTPPSVYPLAPGSAAQTNSMVTLGCLVK + OXIM VNSAAPAPEK		Secreted	
29 (5239)	KAC_MOUSE	P01837	Ig kappa chain C region	91.6 6 29.5	DSTYSMSSTLTLTKDEYER + OXIM HNSYTCEATHK HNSYTCEATHKTSTSPIVK QHQVLTSWTDQDSK SFNRNEC WKIDGSER			

33 (2717)	KNG1_MOUSE	O38677	Kininogen-1 [Precursor]	97.9 17 88.6	ATSQVVAGTKYVIEFIAR AVFPCGCVNAVSTDSPDLEP/LK CQALDMTEMAR CQALDMTEMAR + 2 OxPM CQALDMTEMAR + OxPM DAEEAATGECTATVGK EHEFFIVTQTCK ESNTEAEDCEIK FPSLHGDCVALPHGDDGECR HLGQSLDCNANVYMRPVENIK + OxPM RPPGFSPFR SGNQYMLHR SGNQYMLHR + OxPM SVTYQETK TDGSPTFYSFK VIEGFKTDGSPTFYSFK YVIEFIAR	Kng1 Kng	Secreted Extracellular	(1) Kininogens are inhibitors of thiol proteases; (2) HMW-kininogen plays an important role in blood coagulation by helping to position optimally prekallikrein and factor XI next to factor XII; (3) HMW-kininogen inhibits the thrombin- and plasmin-induced aggregation of thrombocytes; (4) the active peptide bradykinin that is released from HMW-kininogen shows a variety of physiological effects: (4A) influence in smooth muscle contraction, (4B) induction of hypotension, (4C) natriuresis and diuresis, (4D) decrease in blood glucose level, (4E) it is a mediator of inflammation and causes (4E1) increase in vascular permeability, (4E2) stimulation of nociceptors (4E3) release of other mediators of inflammation (e.g. prostaglandins), (4F) it has a cardioprotective effect (directly via bradykinin action, indirectly via endothelium-derived relaxing factor action); (5) LMW-kininogen inhibits the aggregation of thrombocytes; (6) LMW-kininogen is in contrast to HMW-kininogen not involved in blood clotting.
31 (4339)	KV3A4_MOUSE	P33977	Ig kappa chain V-III region 53S13.1	85.7 7 65.7	DIVLTQSPASLAVSLGQR DIVLTQSPASLAVSLGQRATISCR EVPVTFGGGTKLEIK FSGSGSGTDFSLNIHPMEEDDTAMYFCQQSK FSGSGSGTDFSLNIHPMEEDDTAMYFCQQSK + 2 OxPM FSGSGSGTDFSLNIHPMEEDDTAMYFCQQSK + OxPM LLIYAASNQGSGVPAR			
32 (4333)	KV5K_MOUSE	P31644	Ig kappa chain V-V region HP R16.7	84.3 5 59.4	ASQDISNYLNWYQQKPDGTVK DIQMTQTTSSLSASLGDR + OxPM DIQMTQTTSSLSASLGDRVTISCR + OxPM LLIYYTSR TFGGGTKLEIK			Anti-arsonate hybridoma protein.
33 (5131)	KV5L_MOUSE	P31645	Ig kappa chain V-V region HP 93G7	81.3 8 33.6	ASQDISNYLNWYQQKPDGTVK DIQMTQTTSSLSASLGDR DIQMTQTTSSLSASLGDR + OxPM DIQMTQTTSSLSASLGDRVTISCR DIQMTQTTSSLSASLGDRVTISCR + OxPM LLIYYTSR LLIYTSRLHSGVPSR TFGGGTKLEIK			Anti-arsonate hybridoma protein.
34 (8231)	MBL1_MOUSE	P39339	Mannose-binding protein A [Precursor] MBP-A Mannan-binding protein Ra-reactive factor polysaccharide-binding component p28B polypeptide RaRF p28B	96.3 6 74.6	AIEEKLANIMEAEIR + OxPM LANIMEAEIR + OxPM NAEENKAIQEVATGIAPLGITDEATEGQFMYVTGGR + OxPM SLCTELQGTVAIPR SLCTELQGTVAIPRNAEENK VKSLCTELQGTVAIPR	Mbl1	Membrane	Binds mannose and N-acetylglucosamine in a calcium-dependent manner. Is capable of host defense against pathogens, by activating the classical complement pathway independently of the antibody.
35 (4738)	MUC_MOUSE	P31872	Ig mu chain C region secreted form	232.3 18 83.3	DGFSGPAPR DGFSGPAPRK DGKLVESGFTDPYTIENK EFVCTVTHR EQLNLRESATVTCLVK FSKPNEVHKHPAVYLLPPAR GFSPADISVQWLQR GVASVCVEDWNNRK GVASVCVEDWNNRK HPPAVYLLPPAR KEFVCTVTHR LICEATNTPKPTVSWLK LVESGFTDPYTIENK NLVAMGCLAR NLVAMGCLAR + OxPM SLEGSDEVLVCK TGGKVLATSQVLLSPK YLATSQVLLSPK	Igh-6	Secreted	
36 (1117)	MUP8_MOUSE	P24938	Major urinary proteins 11 and 8 [Fragment] MUP11 and MUP8	91.7 7 21.5	AGEYSVTYDGFNTFTIPK EKIEDNGNIFR ENIDLSNANR FAQLCEEHGILR FAQLCEEHGILRENIDLSNANR INGEWHTILASDKR LFLEQNYLEHSLVLK	Mup8 Mup11	Secreted	Binds pheromones that are released from drying urine of males. These pheromones affect the sexual behavior of females.

37 (5827)	PLMN_MOUSE	P22918	Plasminogen [Precursor] EC 3.4.21.7	456.3 39 88.4	AGLEKNYCR CEGETDFVCR CGNVAAAFPNR + OxPM CTTPPPPSPTYQCCL DVILEKR EQDCVIMAENSK FVDWER FVDWEREMR + OxPM GEIVPGSTVSVTVSGK GKTAVTAAGTPCQGWAAQEPHR HSFTPQTNPR KCQSVAAAMFPHR + OxPM LILEPNNR LILEPNNRDALLK LKEAQLPVEPK LSRPATFDK LYDYCDPLCASASSFECGKPQVEPKK MRDVILFEK + OxPM NLEENYCR NPDGDPWGPWCYTTNPR RVYLSECK SFQYHSK SFQYHSKEQQCVIMAENSK + OxPM SSRPEFYK TAVTAAGTPCQGWAAQEPHR TGKDIQDYRGTMSR + OxPM TICYIFQGWGETQGTFGAGR TPENFPCK TPENFPDAGLEMNYCR + OxPM VEYLNNR VILGAHEEYIR VIPACLPSPNYMVADR + OxPM VSRFVDWIER VVGGCVANPHSWPWQISLR YYLSECKTGKGNKGYR WEYCDIPR WEYCNLKR WSEQTPHR WSEQTPHRHNR	Plg	Secreted	Plasmin dissolves the fibrin of blood clots and acts as a proteolytic factor in a variety of other processes including embryonic development, tissue remodeling, tumor invasion, and inflammation; in ovulation it weakens the walls of the Graafian follicle. It activates the urokinase-type plasminogen activator, collagenases and several complement zymogens, such as C1 and C5. It cleaves fibrin, fibronectin, thrombospondin, laminin and von Willebrand factor. Its role in tissue remodeling and tumor invasion may be modulated by CSPG4.
38 (5215)	RETBP_MOUSE	Q33724	Plasma retinol-binding protein [Precursor] PRBP RBP	182.3 13 66.5	ARFSGLWYAIAK DPINGLSPETRR FSGLWYAIAK LQNLDGTCADSYSFVFSR LQNLDGTCADSYSFVFSRDPNGLSPETR MKYWGVASFLQR MKYWGVASFLQR + OxPM QEELCLER QRQEELCLER QYRWEHNGYCQSRPSR WEHNGYCQSRPSR WEHNGYCQSRPSRNSL YWGVASFLQR	Rbp4	Secreted	Delivers retinol from the liver stores to the peripheral tissues. In plasma, the RBP-retinol complex interacts with transthyretin, this prevents its loss by filtration through the kidney glomeruli.
39 (5334)	S6A11_MOUSE	P31653	Sodium- and chloride-dependent GABA transporter 4 GAT4	59.2 5 67.2	AVHERGHWVNIK FPVLCVK LGASPRTVTVNIDCEAK VKQDGTSAITEK VLASDGIEHIGNLR	Gabt4 Gat4 Gat-4	Membrane	Terminates the action of GABA by its high affinity sodium-dependent reuptake into presynaptic terminals. Can also transport beta-alanine and taurine.
40 (8129)	SAA4_MOUSE	P31532	Serum amyloid A-4 protein [Precursor] Amyloid A-S protein	137.3 8 48.3	AVRDHLEAVNQNADQYFYAR EAVQGFWDDLWR GRYEAQQR KVFQGLLNR NHGLETLQATQK NHGLETLQATQKAEEWGR NPIHFRPEGLPEKF YFQGLLNR	Saa4 Saa5	Secreted	Major acute phase reactant. Apolipoprotein of the HDL complex.

41 (7722)	TRFE_MOUSE	Q92111	Serotransferrin [Precursor] Transferrin Siderophilin Beta-1-metal-binding globulin	473.0 42 95.8	A\SSFFSQSCV/PCADP/AFPK CAPHNKEEYNGVTGAFR CPVKLPEGTTPEK CLVDKGDVAFVK DFASCHLAQAPNHVVVSR DLFKDSAFGLLR DLFRDDTK DQYELLCLDNTR DSAFGLLR GTDFQLNQLEGK GTDFQLNQLEGKK GVYAVAVVVK HQFVLDNTEGKNPAEWAK IPSHAVVAR KPVDQYEDCYLAR KPVKDFASCHLAQAPNHVVVSR KSCHTGLGR KSCHTGVDR KTSVPDCIK LCQLCPGCGCSSTQPFFGYVGAFK LLEACTFHKH LPEGTTPEK LVLGHNYVTAIR NILQEDFELLCPDGTR NQQEGYCPEGSIDNSP/K SAGWVIPIGLLFCK SCHTGLGR SCHTGVDR SCHTGVDRTAGWNIPMGMLYNR + OXPM SKDFQLFSSPLGK TAGWNIPMGMLYNR TAGWNIPMGMLYNR + OXPM TSVPDCIK TVLPPDGPR VAQEHFGK VKAVLTSQETLFGGSDCTGNFCLFK VPPRMDYR + OXPM VVCALSHLER VVCAVSEHEATK YLGAEYMQSVGNMR YLGAEYMQSVGNMR + OXPM YLGAEYMQSVGNMRK + OXPM	Tf Trf	Secreted	Transferrins are iron binding transport proteins which can bind two atoms of ferric iron in association with the binding of an anion, usually bicarbonate. It is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization. Serum transferrin may also have a further role in stimulating cell proliferation.
42 (5131)	TTHY_MOUSE	P07309	Transthyretin [Precursor] Prealbumin	136.0 13 63.5	FVEGVYR FVEGVYRVELDTK GSPAVDVAVK HVTIAALSPYSYSTTAVVSNPQN KTSEQSWEPFASGK TAESGELHGLTTDEK TAESGELHGLTTDEKFVEGVYR TLGIGPFHEFADVVFTANDSGHR TSEGSWEPFASGK VLDAVRGSPAVDVAVK	Ttr	Secreted	Thyroid hormone-binding protein. Probably transports thyroxine from the bloodstream to the brain.
43 (3736)	VTDB_MOUSE	P21614	Vitamin D-binding protein [Precursor] DBP Group-specific component Gc-globulin VDB	239.0 24 86.7	CCESTSEDCMASELPEHTIK + OXPM DLCQGSTTQAMDQVTPELSR DLCQGSTTQAMDQVTPELSR + OXPM ECCDTQDSVACFSTQSPLLKR EVVSLTECCAEGADPTCYDTR FSSSTFEQVTNQLVK HLSLLTTMSNR HLSLLTTMSNR + OXPM KFSSTFEQVTNQLVK LAQKVPTADLEDVLPLAEDITEILSR LQMVKHLSLLTTMSNR + 2 OXPM LQMVKHLSLLTTMSNR + OXPM RTQVPEVFLSK SCESDAPFPVHPGTPECCTK SCESDAPFPVHPGTPECCTKEGLER SLSLLVSR SLSLLYSRK SRLSHLIK TQVPEVFLSK VCNELAMLGKEDFR VCNELAMLGKEDFR + OXPM VCSQYAAYGK VCSQYAAYGKEK VLEPTLKTLR	Gc	Secreted	Multifunctional protein found in plasma, ascitic fluid, cerebrospinal fluid, and urine and on the surface of many cell types. In plasma, it carries the vitamin D sterols and prevents polymerization of actin by binding its monomers. DBP associates with membrane-bound immunoglobulin on the surface of B-lymphocytes and with IgG Fc receptor on the membranes of T-lymphocytes.
44 (1714)	VTNC_MOUSE	P29788	Vitronectin [Precursor] Serum-spreading factor S-protein	85.0 13 52.1	EPQFISR FEGVFLDPDYPR GQYCVELDETAVRPGYPK IVVTGSLSHSAQAK LIQDVWGIEGPIDAAFTR NGSLEAFR NWHGVPGKVDAAMAGR NWHGVPGKVDAAMAGR + OXPM SIDGAREPQFISR TVLFKGSQVWR	Vtn	Secreted Extracellular	Vitronectin is a cell adhesion and spreading factor found in serum and tissues. Vitronectin interact with glycosaminoglycans and proteoglycans is recognized by certain members of the integrin family and serves as a cell-to-substrate adhesion molecule. Inhibitor of the membrane-damaging effect of the terminal cytolytic complement pathway.
45 (2535)	ZA2G_MOUSE	Q64726	Zinc-alpha-2-glycoprotein [Precursor] Zn-alpha-2-glycoprotein Zn-alpha-2-GP	93.7 11 60.3	AREEIFLVTLK AVLEECPECPEMLKLR AVLEEECPEMLKLR + OXPM CLAYGFYPQR DTTGSHTFQQMFGCEITNNIR + OXPM GFSQSLSYQWDR IDPPTVTITSR ISLPNVNK KLAFEPER SHLDRIDPPTVTITSR VIPDGRNFK	Azgp1	Secreted	Stimulates lipid degradation in adipocytes and causes the extensive fat losses associated with some advanced cancers
46 (2844)	EGFR_MOUSE	Q01279	Epidermal growth factor receptor	186.0 20 93.9	ACGPDYYEVEEDGIRK ACGPDYYEVEEDGIRK AVNHVCNPLCSSEGCWGPEPR CNLEGEPR GPDNCIQCAHYIDGPHCVK GRSPSDCCHNQCAAGCTGPR IKKAQDCSHR IPLENLQIR NLCYANTINWK NLQELGSAVR NVDLSFLK NYVVTDHGSCVR SLKEDDGDYISGNR SPSDCCHNQCAAGCTGPR TGLRELPMR TPPLDPRELEILK VCTAISGDLHILPVAFK VCTAISGDLHILPVAFKGDSFTR YSFGATCVK YSFGATCVKK	Egfr	Membrane	The EGF receptor mediates the biological signal of EGF, and also of TGF-alpha, amphiregulin, heparin-binding EGF, CRP39 and vaccinia virus growth factor.
47 (7636)	GCBM_MOUSE	P01867	Ig gamma-2B chain C region, membrane-bound form	109.0 11 57.8	APQVYILPPPAEQLSR CPAPNLEGGPSVFIFPPNIKDVLMISLTPK + OXPM HEDLKNYVLK LEPSGPRSTIIPCPPCK LEPSGPRSTIIPCPPCKECHK TDSFSCNVR TDSFSCNVRHEGLK TSRSPGLDLDDICAEAK TTPPSVYPLAPGCGDTTGSSVTLGCLVK VNHKDLPSPIER VNEKTDSFSCNVR		Membrane (Isoform 1) Secreted (Isoform 2)	
48 (1237)	IGJ_MOUSE	P01592	Ig J chain	88.7 6 62.6	CVTTMVPLR + OXPM CVTTMVPLRYHGETK + OXPM IPSTEDPIEDVERR IPSTEDPIEDVERNIR NPVHKLSDVCK NPVYHLSDVCKK	Igj		Serves to link two monomer units of either IgM or IgA. In the case of IgM, the J chain-joined dimer is a nucleating unit for the IgM pentamer, and in the case of IgA it induces larger polymers. It also help to bind these immunoglobulins to secretory component.
49 (2188)	KV3A3_MOUSE	P01656	Ig kappa chain V-III region MOPC 73	73.5 5 61.6	DIVLTQSPASLAVSLGQR DIVLTQSPASLAVSLGQRATISCR EVPWTFGGGTKLEIK FSGSGSDTDFSLNIHPMEEDDTAMYFCQQSK + 2 OXPM LLIYAASNQGSGVPAR			Bence-Jones protein
50 (7733)	PROP_MOUSE	P11683	Properdin	92.2 9 73.4	CGGHCPGEAQQSQACDTQK HGGPFCAGDATR LVVEEKR MSINCEGTPQQQSR MSINCEGTPQQQSR + OXPM QRLCTPLVPK QRYCDNPAPK SCSAPAPSHQPPGKPCSGPAYEHK TCDHPAPR	Cfp Pfc	Secreted	A positive regulator of the alternate pathway of complement. It binds to and stabilizes the C3- and C5-convertase enzyme complexes

Publikation II:

A 2-DE MALDI-TOF study to identify disease regulated serum proteins in lung cancer of c-myc transgenic mice

Published in: *Proteomics* 2009, *9*, 1044-1056

Weblink: http://www3.interscience.wiley.com/journal/121670156/abstract

Bijon Chatterji [1], Jürgen Borlak [1, 2]

[1] Department of Drug Research and Medical Biotechnology, Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM), Hannover, Germany
[2] Center for Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

Corresponding author:

Prof. Dr. J. Borlak
Department of Drug Research and Medical Biotechnology
Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM)
Nikolai-Fuchs-Str. 1
D-30625 Hannover, Germany
Phone: +49-511-5350-559
Fax: +49-511-5350-573
E-mail: borlak@item.fraunhofer.de

Keywords:

Serum / c-myc / Lung cancer / Biomarkers / 2-DE / MALDI-TOF/TOF

Abstract

We previously reported targeted overexpression of c-myc to alveolar epithelium to cause lung cancer. We now extended our studies to the serum proteome of tumor bearing mice. Proteins were extracted with a thiourea-containing lysis buffer and separated by 2-DE at pH 4-7 and 3-10 followed by MALDI-TOF/TOF analysis. Forty six proteins were identified in tumor bearing mice of which n=9 were statistically significant. This included disease regulated expression of orosomucoid-8, alpha-2-macroglobulin, apolipoprotein-A1, apolipoprotein-C3, glutathione peroxidase-3, plasma retinol-binding protein and transthyretin, while expression of apolipoprotein-E was decreased at late stages of disease. Moreover, serum amyloid P component was uniquely expressed at late stages of cancer. It is of considerable importance that most disease regulated proteins carried the E-Box sequence (CACGTG) in the promoter of the coding gene, therefore providing evidence for their regulation by c-myc. Notably, expression of alpha-2-macroglobulin, transthyretin, alpha-1-antitrypsin and properdin was in common in different lung tumor models, but regulation of orosomucoid-8, apolipoprotein-A1, apolipoprotein-C3, apolipoprotein-E, glutathione peroxidase-3, plasma retinol-binding protein and serum amyloid P component was unique when the serum proteome of c-myc and c-raf tumor bearing mice were compared. Therefore, candidate biomarkers to differentiate between atypical adenomatous hyperplasias (AAH) and bronchiolo-alveolar (BAC)/papillary adenocarcinomas (PLAC) can be proposed.

1 Introduction

Lung cancer remains the leading cause of cancer death worldwide. In 2007, approximately 160,000 people died from lung cancer in the United States alone (American Cancer Society, 2007). Smoking is considered to be the primary cause of lung cancer and accounts for > 80% of all diagnosed cases [1].

In general, lung tumors are classified as small cell (SCLC) or non-small cell lung carcinomas (NSCLC). NSCLC are further divided into adenocarcinomas, large and squamous cell carcinomas. Subclasses of adenocarcinomas may be divided further in Clara and alveolar epithelial cancers [2]. Indeed, a recent study suggests alveolar epithelial carcinomas to be on the rise and may account for up to one third of all adenocarcinomas [3].

The majority of cancers are considered to be of sporadic origin, but many tumors display altered c-myc activity. Notably, c-myc becomes overexpressed or amplified in human lung cancer cells [4-15]. For instance, to determine the role of oncogene overexpression in NSCLC, c-myc was analyzed in surgically or endoscopically obtained tumor samples and samples of normal bronchopulmonary tissue taken from the same patients [10]. In contrast to normal tissue, there was up to 57-fold increase in c-myc mRNA in tumors. Furthermore, c-myc overexpression was significantly more prevalent in large-cell cancer than in adenocarcinoma, and tumor differentiation was negatively correlated with c-myc mRNA amounts. Recently, a study examined >150 human NSCLCs and showed that Wnt1 overexpression was associated with tumor proliferation and poor prognosis in lung cancer patients [15]. Since c-myc is one of the proliferation-regulating targets of Wnt, its expression was analyzed as well. Indeed, the percentage of lung tumor cells expressing c-myc was significantly higher in Wnt-positive than in Wnt-negative tumors.

However, as early detection of disease improves overall survival, we were particularly interested to study disease regulated proteins. Here we report our

efforts to identify serum biomarkers of disease in a c-myc transgenic lung cancer mouse model as previously reported by us [16].

Specifically, c-myc encodes a 49 kDa nuclear phosphoprotein that is classified as a basic helix-loop-helix/leucine zipper-type (bHLH/LZ) transcription factor. C-myc progresses cell cycle regulation [17] and forms a heterodimeric protein complex with max, another 18 kDa bHLH/LZ protein. The c-myc/max complex then recognizes the consensus sequence 5'-CACGTG-3', also known as an E-box motif [18]. This motif is located in promoter sequences of many genes targeted by c-myc. Genome wide scanning by DNA microarray, SAGE and chromatin immunoprecipitation (ChIP) provide evidence for more than 1,000 c-myc target genes. Through its numerous direct and indirect targeted genes, the c-myc oncoprotein is linked to many cellular processes including signal transduction, DNA synthesis and repair, apoptosis, cell adhesion, cytoskeleton dynamics and regulation of ion channels [19]. As detailed above, expression and activity of c-myc is increased in many human malignancies either due to undue amplification of the reference allele or by hyperactivity of the mutated protein [20]. Nonetheless, the precise molecular mechanism by which c-myc influences cell growth and division in normal and tumorous cells remains elusive.

In fact, in the United States alone more than 70.000 cancer deaths per year are ascribed to altered c-myc abnormalities [21]. In the past we reported targeted overexpression of c-myc to alveolar epithelium to result in bronchiolo-alveolar carcinomas (BAC) and papillary adenocarcinomas (PLAC) [16, 22]. As of today, disease regulated serum proteins of c-myc-induced lung adenocarcinomas are unknown. We therefore applied 2-DE and MALDI MS to identify candidate biomarkers at early and late stages of disease. We further compared our findings with results from a recently published serum proteome study with c-raf transgenic mice, which developed lung cancer as well [23]. Thus, serum biomarkers may be identified to differentiate amongst different lung cancer malignancies. Overall, this study aimed at identifying candidate disease biomarkers for the detection of lung cancer.

2 Materials and methods

2.1 Histopathology

Transgenic lung tumor bearing mice were bred and kept in the C57BL/6 background. A detailed description of the SP-C/c-myc transgenic line is given in [16]. Here we refer to C57BL/6 non-transgenic littermates as controls. Fig. 1a depicts the SP-C/c-myc gene construct while Fig. 1b (B1, B2) displays typical features of the lung tumors. The mouse tumors were classified according to the IARC - WHO system of classification (2004) by the board certified pathologist Dr. Reinhard Spanel (Leipzig, Germany). Different stages of tumor development in the alveoli were observed. Early stages were characterized by multifocal displastic changes originating in the alveolar epithelium. Multifocal BAC were detected on average around 8-10 months. Typically animals displayed PLAC around 12-14 months with metastatic spread to liver and other organs. Tissues were fixed in 4% buffered formaldehyde in PBS for approximately 20 h, dehydrated and embedded in paraffin (Roti-Plast ™, Roth). Tissue sections were stained with hematoxylin and eosin according to standard protocols.

2.2 Sample preparation

Blood serum of healthy aged matched controls (n=12) and of lung tumor bearing SP-C/c-myc mice, aged 3 (n=6) and 14 months (n=6) were studied. Blood was withdrawn from the *vena cava*. After clotting for 2 h at room temperature, the blood was centrifuged at 3500 rpm for 15 min. The resultant supernatants were removed, frozen immediately in liquid nitrogen and stored at -80°C until further analysis. The protein concentration in serum was determined by the Bradford protein assay.

2.3 Two-dimensional gel-electrophoresis

Each sample was analyzed in triplicate. Serum proteins were separated without

any pre-treatment by isoelectric focusing (IEF) with precast IPG strips (pH 3-10, non-linear gradient and pH 4-7, linear gradient; both 170x3x0.5 mm, BioRad). 800 µg was diluted in a lysis buffer containing 2 mol/L thiourea, 5 mol/L urea, 40 mmol/L Tris, 4% CHAPS, 0.5% BioLyte 3-10 (BioRad), 100 mmol/L DTT [24, 25] resulting in a total volume of 350 µL per strip. IPG strips were rehydrated at 50 V for 12 h. IEF was performed at 20°C with a maximum voltage of 10 kV and a maximum current of 50 µA per strip. After IEF, IPG strips were equilibrated in 10 mL reducing buffer (2% DTT in 10 mL equilibration buffer containing 6 mol/L urea, 30% glycerin, 2% SDS, 0.05 mol/L Tris-HCl, pH 8.8 and 0.5% bromphenol blue) for 15 min, followed by equilibration in 10 mL alkylation buffer (4% iodoacetamide and 0.5% bromphenol blue in 10 mL equilibration buffer) for 15 min [26]. SDS-PAGE was performed in a Protean-plus Dodeca™ Cell (BioRad) using self-cast polyacrylamide gels (200 x 205 x 1.5 mm; 12% T). Gels were run in parallel in 0.025 mol/L Tris/ 0.192 mol/L glycine/ 0.1% SDS at 10°C with a constant voltage of 70 V overnight. Precision Plus Protein Unstained Standards™ (BioRad) was used for calibration of M_r and p/.

2.4 Staining and imaging

2-D Gels were fixed overnight in 500 mL 30% ethanol/ 2% phosphoric acid and washed three times for 20 min each in 500 mL 2% phosphoric acid. Equilibration was done with 500 mL 2% phosphoric acid/ 18% ethanol/ 15% ammonium sulfate thereafter. Colloidal Coomassie Brilliant Blue (CBB) staining of proteins was started by addition of 5 mL staining solution (2% CBB G250, Roth) to 500 mL of equilibration solution. Staining was carried out for 48 h and thereafter washed once with 500 mL water for 10 min. Gels were scanned with the Perfection 4990 Photo™ densitometer (Epson). Detection of spots, quantification and comparison of 2-D protein profiles was done with the PDQuest 8.0 software (BioRad). After removal of background and vertical streaks from each gel image, spots were digitized by Gaussian fit. To quantify protein spots, a matchset of all gels was made and the absorbance of individual protein spots from 2-D gels was measured. The raw quantity of each spot in a member gel was divided by the total intensity

value of all the pixels in the gel image, that is total density. This normalization procedure of the software assumed that the total density of an image, consisting of background and spot density will be relatively consistent from gel to gel [27]. The expression of serum proteins was analyzed by the Student's t-test. A probability of $p<0.05$ was considered statistically significant (Tab. 2a and Tab. 2b). Graphical evaluation was performed with the SigmaPlot software (SPSS) (Fig. 3b). Spots were excised and transferred to 96-well microtiter plates (ABgene) by the EXQuest™ spot cutter (BioRad).

2.5 Mass spectrometry

2.5.1 In-gel digestion

Each of the CBB-stained gel plugs was washed twice with 15 µL ammonium hydrogencarbonate solution (100 mmol/L) and then dehydrated twice with 15 µL acetonitrile. Proteins were digested with a total of 160 µg trypsin (13 ng/µL, sequencing grade, Promega) per gel plug at 37°C for 4 h. Resulting peptides were extracted with 8 µL n-Octyl-ß-D-glucopyranoside (5 mmol/L, Applichem)/ 1% trifluoroacetic acid in an ultrasonic bath (Sonorex, Super RK 514 BH, Bandelin) for 5 min.

2.5.2 MALDI-TOF/TOF

The HCCA matrix was prepared with the thin layer method. 1 µL of the peptide extracts were manually spotted onto a 600 µm/384 well AnchorChip™ sample target (Bruker Daltonics) and dried at ambient temperature. Recristallization was performed with 1 µL of 60% ethanol/ 30% acetone/ 10% of 1% trifluoroacetic acid thereafter. MALDI mass spectra were recorded using an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 384-sample scout source. A peptide calibration standard (Bruker Daltonics) was used for external calibration. MS and MS/MS data were recorded automatically on the MALDI-TOF/TOF instrument using the three most abundant peptide signals of the corresponding

peptide mass fingerprint (PMF) spectrum. Mass spectra were acquired in an automatic mode using the AutoXecute module of FlexControl 2.4 software (Bruker Daltonics). Spectra were analyzed using the FlexAnalysis 2.4 software (Bruker Daltonics). The Swiss-Prot database employing the Mascot 2.0 program (Matrix Science, in-house server) was used for the search of peptide masses to identify proteins [28]. Database searches were performed taking into account carbamidomethyl modification of cysteines and possible oxidation of methionine. One missed cleavage was allowed. A mass accuracy of ≤ 100 ppm was requested for PMF. For MS/MS searches, a mass accuracy of ≤ 70 ppm was allowed for peptide masses and their fragments, respectively. For further consideration, only those proteins were assumed to be identified that were annotated from corresponding spots in at least five gels. Identified proteins were sent to the Proteinscape ™ database (Protagen) [29] and checked individually for further consideration.

2.6 Western Blot

Some disease regulated proteins were also confirmed by Western immunoblotting. Protein samples (50 µg) used in 2-DE were run on 12% SDS-PAGE, blotted onto PVDF membranes and subsequently blocked with TBS/10% Rotiblock™ (Roth) for 1 hour at room temperature. Primary antibodies (Santa Cruz) were diluted in blocking buffer (TBS/1% Rotiblock), added as follows: APOA1, 1:200; APOE, 1:200; RETBP, 1:200; SAMP, 1:200. ß-Tubulin (1:200) was used as loading control. Anti-rabbit (APOA1, RETBP, SAMP), anti-goat (APOE) and anti-mouse (ß-Tubulin) HRP-conjugated IgGs were used as secondary antibodies. Rat liver total extract was used as positive control. Membranes were washed with TBS/0.1% Tween and detected with enhanced chemiluminescence (Perkin Elmer) for 60 min with a CF440 imager (Kodak). Fig. 4 depicts a part of the image with protein bands of interest cropped and marked by molecular weights.

3 Results and Discussion

3.1 Separation of serum proteins by 2-DE

We used a thiourea-containing lysis buffer to extract proteins from serum. Proteins were separated within pH ranges of 3-10 and 4-7, respectively. Proteins were visualized with the colloidal CBB (CCB) stain. Approximately 400 (pH 3-10) and 200 (pH 4-7) spots per gel were detected. Fig. 2a and Fig. 2b depict examples of serum proteome maps (pH 3-10 and 4-7) of c-myc tumor bearing mice at late stages of carcinogenesis (14 months).

Notably, serum proteome profiling is challenging, because of interference by high-abundant proteins such as albumin, antitrypsin, immunoglobulins and transferrin. In particular, pre-fractionation techniques such depletion of serum albumin are useful procedures in proteome profiling studies, but they may introduce bias as well. For instance, pre-fractionation increases the risk of depletion of low-abundant proteins as it has been shown for paraneoplastic antigen MA I, coagulation factor VII precursor, prostate-specific antigen, as a result of multiple protein-protein interactions with IgG, transferrins, and/or gelsolin. To account for the possibilities we did not deplete high-abundant proteins.

3.2 Protein identification

One hundred and eight 2-D gels were stained with the CCB method and studied by image analysis. After excision of >12.500 protein spots tryptic digests were analyzed by MALDI-MS and -MS/MS. Identification was based on Swiss-Prot database entries with the Mascot search engine. In tumor bearing and healthy non-transgenic mice, 46 common serum proteins [30, 31] were identified (Tab. 1). In Supplementary Table 1 we list the Mascot score, sequence coverage and the most informative peptide sequences identified by MS and -MS/MS, respectively.

3.3 Regulation of serum proteins in lung cancer

Tab. 2a and 2b provide expression profiles of statistically significantly regulated proteins ($p<0.05$) in lung tumor serum proteomes. N=13 proteins were regulated at late stages of cancer (14 months), of which n=9 were regulated at early stages of tumorigenesis (3 months) as well. Fig. 3a depicts prominent examples of disease regulated proteins in tumor bearing mice, while Fig. 3b compares the fold-changes between early and late stages of tumorigenesis. Expression of selected proteins was also confirmed by Western blotting (Fig. 4). Differentially expressed proteins will be discussed later for their proven or infered association with human disease. Notably, their regulation by c-myc was determined by comparison of database entries from http://www.myccancergene.org [32] and by searching for the DNA consensus sequence for the E-Box motif 5'-CACGTG-3' in the promoter regions of the coding gene. Overall, six regulated proteins emerged as direct c-myc targets (Tab. 3). These include A1AG8 (spot no. 1), alpha-1-antitrypsin 1-1 (A1AT1, spot no. 2), alpha-1-antitrypsin 1-6 (A1AT6, spot no. 3), alpha-2-macroglobulin (A2M, spot no. 4), apolipoprotein E (APOE, spot no. 12) and glutathione peroxidase 3 (GPX3, spot no. 21). Furthermore, we compared our results with our recently published study on the serum proteome of c-raf transgenic mice which developed lung tumors as well [23]. In the following sections the biological relevance of the regulated proteins is described.

3.3.1 Regulation of acute phase proteins in lung cancer

In general, concentrations of various positive and negative regulators of acute phase proteins (APP) increase or decrease in response to inflammation [33]. As observed in serum of healthy and tumor bearing mice, seven acute phase proteins were regulated. We found the positive acute phase regulators, i.e. A1AG8 (spot no. 1), A1AT1 (spot no. 2) and A1AT6 (spot no. 3), A2M (spot no. 4-B) and serum amyloid P component (SAMP, spot no. 41) to be increased or exclusively expressed in tumor bearing mice. In contrast, the negative acute phase proteins (n-APP) plasma retinol-binding protein (RETBP, spot no. 40) and transthyretin (TTHY, spot no. 44) were upregulated (see Figures 3a, 3b and Tables 2a, 2b).

Specifically, **A2M** (spot no. 4, 4-B, 4-C, 4-D) is mainly produced in the liver, but in the lung as well. With a molecular weight of 165 kDa, A2M represents a large plasma protein that consists of four identical subunits that are linked together by disulfide bonds. A2M acts as a proteinase inhibitor and targets serine-, cysteine-, aspartic- and metalloproteinases. The A2M structure includes a "decoy" region where such proteinases are bound and cleaved. Macrophage receptors recognize and eliminate the A2M-proteinase complex. For instance, it is known that A2M is regulated in patients diagnosed with a nephrotic syndrome as well as Alzheimer's disease. A determination of serum A2M levels may thus be extended to the early detection and therapeutic monitoring in lung cancer [23] and in bone metastases of prostate cancer as reported elsewhere [34]. In a previous study the responsiveness of A2M to c-myc regulation was shown by Misra and coworkers [35]. We observed a significant ($p<0.05$), > 6-fold (pH 4-7) and 1.5-fold (pH 3-10) increase of A2M levels in serum of tumor bearing mice, aged 14 months and a 2.7-fold change in tumor bearing mice, aged 3 months (spot no. 4-B).

Furthermore, **SAMP** (spot no. 41), a member of the pentraxins that is produced in the liver, was marginally increased ($p>0.05$) at early stages, but exclusively expressed at late stages of tumorigenesis in our transgenic disease model. SAMP has a sequence homology of 51% with CRP (spot no. 18), a well known plasma APP. Although the physiological role of SAMP is not always clear, it may play a role in amyloidosis [36]. Recently, Korbelik and co-workers reported significant increase of SAMP levels in patients with liver tumors [37]. We observed exclusive expression of SAMP at later stages of tumorigenesis as confirmed by Western blotting (Fig. 4).

We also observed regulation of **TTHY**, also named prealbumin (spot no. 44). This protein is a carrier for thyroid hormones from bloodstream to tissues. TTHY interacts with RETBP (spot no. 40), thus enabling retinol transportation. Lower levels of TTHY coincide with lower levels of retinol and RETBP, as reported for ovarian cancer [38, 39]. Notably, a truncated form of TTHY was found to be repressed in women with ovarian cancer [40]; this protein may therefore serve as a serum biomarker of disease [41]. A decrease in TTHY serum levels is also seen in negative acute phase regulation during inflammation; correspondingly its

increased expression might be utilized as a biomarker for cancer. The TTHY monomer may also serve as a marker to cerebrospinal fluid barrier disruption, as shown in cerebral metastasis and lung cancer [42], even though it is differentially expressed in tumors [43]. In a recent proteomic study induction of TTHY in human lung adenocarcinoma patients was reported. We also observed upregulation of TTHY by >1.4-fold in serum of lung tumor bearing mice, alongside an overexpression of RETBP at early and late stages of tumorigenesis by 2.5-fold (pH 4-7) and 5-fold (pH 3-10), respectively.

3.3.2 Regulation of apolipoproteins in lung cancer

We found several apolipoproteins to be regulated in tumor bearing mice. These proteins function primarily as lipid-binding proteins [44] to transport lipids from the intestine to the liver and from the liver to tissues, including adipocytes, lung, heart, muscle and breast tissues. As apolipoproteins are detergent-like, they solubilize the hydrophobic parts of lipoproteins. Additionally, they are widely known as receptor ligands, enzyme co-factors and lipid carriers that are involved in regulation of the intravascular metabolism of prior to their ultimate tissue uptake. The synthesis of apolipoproteins is controlled by hormones such as insulin or glucagon and environmental factors including alcohol and intake of drugs, such as fibric acids, statins or niacins. It is of considerable importance that regulation of subclasses of apolipoproteins are reported for a number of malignancies [45-47]. Here we evidence differential expression of apolipoproteins A, C, E and H at different stages of tumor development in c-myc transgenic mice. Their disease association is discussed below.

Specifically, **apolipoprotein A-I** (APOA1, spot no. 9) belongs to the ApoA1/A4/E protein family and is primarily produced in the liver and the intestine. APOA1 can be found in the extracellular space and, being a structural component of high density lipid proteins (HDL), takes part in cholesterol absorption. APOA1 upregulation is associated with breast and lung cancer as suggested elsewhere [48, 49]. Here we report APOA1 expression to be significantly increased by 1.8-fold in tumor bearing mice, aged 3 months and by 1.4-fold (pH 4-7) and 2.8-fold

(pH 3-10) in tumor bearing mice, aged 14 months.

Spot no. 11 was identified as **apolipoprotein C3** (APOC3) which is produced in the liver. This protein inhibits the lipoprotein and hepatic lipase and represses the uptake of lymph chylomicrons by hepatic cells. Thus, APOC3 may repress the catabolism of triglyceride-rich particles. Upregulation of APOC3 was demonstrated in a chronic renal failure model [50] and in diabetes [51], but regulation of APOC3 in lung cancer was not reported so far. Serum levels of APOC3 were increased already at early stages of cancerogenesis by 2.3-fold and still increased by 2.7-fold (pH 4-7) and 1.7-fold (pH 3-10) in lung tumor bearing mice, aged 14 months.

APOE (spot no. 12) is a mediator for binding, internalizing and metabolism of lipoprotein particles. It serves as a ligand for the low density lipoprotein (LDL) receptor and for the APOE receptor (chylomicron remnant) of hepatic tissues. APOE expression was marginally induced ($p<0.05$) in serum of tumor bearing mice, aged 3 months, but reduced in tumor bearing mice, aged 14 months, by 1.6-fold (pH 4-7) and 1.2-fold (pH 3-10), respectively. Regulation of APOE was reported for human hepatocellular [52], colorectal [53] and pancreatic carcinoma [54]. Its regulation in lung cancer is novel and was not reported so far. Notably, APOE is reported to modulate clearance of apoptotic bodies *in vitro* and *in vivo* [55] and therefore may play a role in various pathologies. *In silico*, we identified an E-Box motif in the promoter region of the APOE gene therefore suggesting APOE to be a direct target of c-myc.

Additionally, **apolipoprotein H**, also named as beta-2 glycoprotein-1 (APOH, spot no. 13) binds to various kinds of negatively charged substances such as heparin, phospholipids and dextran sulfate. Through binding to phospholipids on the surface of damaged cells, APOH may inhibit activation of the intrinsic blood coagulation cascade. APOH is synthesized in the liver and secreted into plasma. As reported by others, APOH induction may be involved in blocking angiogenic processes in bladder cancer [56]. APOH expression was induced in sera of lung tumor bearing mice by 1.4-fold (pH 4-7) and 1.7-fold (pH 3-10) at late stages of

lung cancer while its expression was unchanged at early stages.

3.3.3 Oxidative defense and complement activation in lung cancer

GPX3 (spot no. 21) functions in response to oxidative damage by catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide. Induction of GPX3 expression was observed in ovarian cancer [57] and diabetes [58]. We observed an initial upregulation of GPX3 in serum of lung tumor bearing mice, aged 3 months, by 1.7-fold, followed by an increase in transgenic mice, aged 14 months, by > 10-fold. Note, the E-Box motif "CACGTG" in the promoter region of the GPX3 gene makes this gene a likely target for c-myc.

Furthermore, **properdin** (PROP, spot no. 39), also known as factor P, is a serum glycoprotein and positive regulator of the alternate pathway for complement activation. It binds to and stabilizes the C3- and C5-convertase enzyme complexes. Complement C3 (CO3, spot no. 17) was not regulated in tumor bearing mice whereas PROP expression was increased by 1.5-fold at late stages of tumorigenesis only. PROP plays a role in some specific immune responses and in tissue inflammation. It is known that PROP is involved in engulfing of pathogenes by phagocytes thereby helping to neutralize some viruses, such as the influenza virus [59, 60].

3.4 Disease associated regulation of serum proteins in c-myc and c-raf transgenic mouse models of lung cancer

We recently reported the serum proteome mapping of c-raf transgenic mice which developed lung cancer through targeted overexpression of the serine/threonine kinase to alveolar epithelium [23]. Because of distinct differences in the lung tumor pathologies, i.e. AAH versus BAC and PLAC we wished to compare regulation of serum proteins in c-myc and c-raf transgenic mice. Strikingly, our data are highly suggestive for candidate serum biomarkers that enable differentiation between AAH and BAC as observed in these two different transgenic disease models.

Table 3 gives an overview of our findings. Notably, A1AT6, A2M, TTHY and PROP were commonly regulated in c-myc and c-raf transgenic mice and thus may serve as general serum biomarkers of disease. In c-myc transgenic mice apolipoproteins, RETBP or SAMP were exclusively associated with BAC. Likewise, the regulation of major urinary proteins (MUP), vitamin D-binding protein (VTDB, spot no. 45) or a soluble form of the epidermal growth factor receptor (EGFR) was uniquely associated with AAH in c-raf transgenic mice.

4 Conclusions

Based on our previous work on the SP-C/c-myc transgenic mouse model [16] we now report disease regulated serum proteins at early and advanced stages of lung cancer. Some of the identified proteins are acute phase reactants (APP) and were either differentially regulated or exclusively expressed in lung cancer bearing mice, as shown for A1AG8, A1AT1, A1AT6, A2M, SAMP, RETBP and TTHY. For some of the identified proteins A2M and APOA1 regulation in lung cancer has been reported [49], while disease regulation of APOE is novel and may play a much broader role in cancer [55]. Additionally, we observed disease associated regulation of APOH which has been linked to angiogenesis as well [56]. We also report GPX3, PROP and TTHY to be upregulated in tumor bearing mice, some of which are direct c-myc targets such as GPX3. This antioxidant was shown to be regulated in ovarian cancer as well [57] while an identification of PROP may be in response to tumor associated inflammation. Finally, increased TTHY serum levels observed in tumor bearing mice was also reported for human lung cancer patients [42]. While a number of differentially expressed proteins display the E-Box binding sites in the promoters of the coding gene, i.e. A1AG8, A2M, APOE and GPX3, their regulation as a consequence of altered c-myc activity remains elusive. Notably, decreased expression of GPX was shown in another c-myc transgenic mouse model [61], but further investigations are needed to determine the c-myc regulation of the herein described regulated proteins. Some of the disease regulated proteins are not direct targets of the c-myc transcription factor, i.e. several apolipoproteins, such as PROP, RETBP, SAMP and TTHY. Based on two different models of lung cancer we propose serum candidate biomarkers to differentiate between atypical adenomatous hyperplasia (AAH) and bronchiolo-alveolar (BAC)/papillary adenocarcinomas (PLAC) at early stages of disease.

Figure captions

Figure 1a:

Gene construct of the SP-C/c-myc transgenic mouse model as reported by Ehrhardt *et al.* [16].

Figure 1b:

Histology of a lung of a healthy (A1) and a tumor bearing (B1) SP-C/c-myc transgenic mouse, aged 14 months. Hematoxylin and eosin staining was used for histopathology of tumors. A2 and B2: macroscopical views of lungs of healthy and tumor bearing mice, respectively.

Figure 2a:

2-DE serum proteome map of lung tumor bearing SP-C/c-myc transgenic mice (14 months) with a pH range from 3-10.

Figure 2b:

2-DE serum proteome map of lung tumor bearing SP-C/c-myc transgenic mice (14 months) with a pH range from 4-7.

Figure 3a:

Examples of regulated serum proteins at different stages of tumorigenesis (3 months and 14 months) in SP-C/c-myc transgenic (T) and healthy (C) mice. Protein spots of interest are marked by circles.

Figure 3b:

Comparison of fold-changes of disease regulated proteins between two stages of lung tumorigenesis, i.e. 3 and 14 months ($p<0.05$).

Figure 4:

Western Blot analysis of some disease regulated proteins. Notably, serum amyloid P component (SAMP, spot no. 41) was exclusively expressed in SP-C/c-myc lung tumor bearing mice at late stages of tumorigenesis (14 months). ß-Tubulin expression was used as the loading control.

Table captions

Table 1:

Protein identification in 2-DE maps of mouse serum proteins including healthy and lung tumor bearing mice, identified by MALDI MS/MS. A total of 46 proteins were identified. *PI* and M_r are based on the theoretical values of the precursor. Sequence coverage and no. of matched peptides: The value from one typical spot was given if many spots were assigned as one number (protein). Protein identification was based on PMF and PFF. See Supplementary Table 1 for detailed information.

Table 2a and 2b:

Expression profiles of significantly regulated proteins ($p<0.05$) from 2-D gels of tumor bearing mice, aged 3 months (pH 3-10) and tumor bearing mice, aged 14 months (pH 4-7 and pH 3-10). Quantification of protein abundance was done using the PDQuest 2-D software (BioRad) by measurement of the normalized optical density (arbitrary units, AU) of each protein spot. The change in abundance of the proteins is expressed by the calculated ratio (T/C) between mean values

from tumor (T) and healthy (C) samples. %RSD: percental relative standard deviation. Spot no. 41 (SAMP) is exclusively expressed in tumor bearing mice. Student's t-test was used for calculation of p-values.

Table 3:

Commonly and specifically regulated proteins in adenomatous hyperplasia (AAH) and bronchiolo-alveolar adenocarcinomas (BAC). Recently, AAH was studied in SP-C/c-raf transgenic mice [23], whereas BAC is the subject of our present work on SP-C/c-myc transgenic mice. 1M: tumor bearing mice, aged 1 month; 12M: 12 months; 3M: 3 months; 14M: 14 months; C: exclusively expressed in control mice; T: exclusively expressed in tumor bearing mice; -: protein not detected; ↔: no regulation/no fold-change; ↑: ≤ 1.5 fold-change; ↑↑: ≤ 3.0 fold-change; ↑↑↑: > 3.0 fold-change; ↓: ≥ 0.7 fold-change; ↓↓: ≥ 0.3 fold-change ; ↓↓↓: < 0.3 fold-change. APP: acute phase protein; E-Box: containing 5'-CACGTG-3' consensus sequence in promoter region.

Supplementary Table 1:

A summary of serum proteins from SP-C/c-myc transgenic mice. The Mascot score (PMF), ions score (PFF), the number of identified peptides, their sequence, the protein coverage of the best hits and supporting information are shown for each identified protein. O@M: oxidation at the amino acid methionine.

Acknowledgements

The authors thank Dr. Roman Halter for providing transgenic mice, furthermore Ignazio Garaguso, Maria Stella Ritorto, Dr. Sven Schuchardt, Dr. Susanne Reymann and Milani Deb for their valuable comments.

The authors have declared no conflict of interest.

5 References

[1] Duarte RL, Paschoal ME. Molecular markers in lung cancer: prognostic role and relationship to smoking. *J Bras Pneumol* 2006, 32, 56-65. *Review*.

[2] Bhattacharjee A, Richards WG, Staunton J, Li C *et al.* Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci USA* 2001, 98, 13790-13795.

[3] Takezawa C, Takahashi H, Fujishima T, Shiratori M *et al.*, Assessment of differentiation in adenocarcinoma cells from pleural effusion by peripheral airway cell markers and their diagnostic values. *Lung Cancer* 2002, 38, 273-281.

[4] Fields WR, Desiderio JG, Putnam KP, Bombick DW, Doolittle DJ. Quantification of changes in c-myc mRNA levels in normal human bronchial epithelial (NHBE) and lung adenocarcinoma (A549) cells following chemical treatment. *Toxicol Sci* 2001, 63, 107-114.

[5] Broers JL, Viallet J, Jensen SM, Pass H *et al.* Expression of c-myc in progenitor cells of the bronchopulmonary epithelium and in a large number of non-small cell lung cancers. *Am J Respir Cell Mol Biol* 1993, 9, 33-43.

[6] Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature* 1983, 306, 194-196.

[7] Zajac-Kaye M. Myc oncogene: a key component in cell cycle regulation and its implication for lung cancer. *Lung Cancer* 2001, 34, S43-S46.

[8] Bernasconi NL, Wormhoudt TA, Laird-Offringa IA. Post-transcriptional deregulation of myc genes in lung cancer cell lines. *Am J Respir Cell Mol Biol* 2000, 23, 560-565.

[9] Barr LF, Campbell SE, Bochner BS, Dang CV. Association of the decreased expression of alpha3beta1 integrin with the altered cell: environmental interactions and enhanced soft agar cloning ability of c-myc-overexpressing small cell lung cancer cells. *Cancer Res* 1998, 58, 5537-5545.

[10] Lorenz J, Friedberg T, Paulus R, Oesch F, Ferlinz R. Oncogene overexpression

in non-small-cell lung cancer tissue: prevalence and clinicopathological significance. *Clin Investig* 1994, 72, 156-163.

[11] Volm M, Drings P, Wodrich W, van Kaick G. Expression of oncoproteins in primary human non-small cell lung cancer and incidence of metastases. *Clin Exp Metastasis* 1993, 11, 325-329.

[12] Pfeifer AM, Mark GE 3rd, Malan-Shibley L, Graziano S *et al.* Cooperation of c-raf-1 and c-myc protooncogenes in the neoplastic transformation of simian virus 40 large tumor antigen-immortalized human bronchial epithelial cells. *Proc Natl Acad Sci U S A* 1989, 86, 10075-10079.

[13] Johnson BE, Battey J, Linnoila I, Becker KL *et al*. Changes in the phenotype of human small cell lung cancer cell lines after transfection and expression of the c-myc proto-oncogene. *J Clin Invest* 1986, 78, 525-532.

[14] Volm M, van Kaick G, Mattern J. Analysis of c-fos, c-jun, c-erbB1, c-erbB2 and c-myc in primary lung carcinomas and their lymph node metastases. *Clin Exp Metastasis* 1994, 12, 329-334.

[15] Nakashima T, Liu D, Nakano J, Ishikawa S *et al*. Wnt1 overexpression associated with tumor proliferation and a poor prognosis in non-small cell lung cancer patients. *Oncol Rep* 2008, 19, 203-209.

[16] Ehrhardt A, Bartels T, Geick A, Klocke R *et al*. Development of pulmonary bronchiolo-alveolar adenocarcinomas in transgenic mice overexpressing murine c-myc and epidermal growth factor in alveolar type II pneumocytes. Br J Cancer 2001, 84, 813-818.

[17] Facchini LM, Penn LZ. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J 1998, 12, 633-651.

[18] Amati B, Frank SR, Donjerkovic D, Taubert S. Function of the c-Myc oncoprotein in chromatin remodeling and transcription. Biochim Biophys Acta 2001, 1471, 135-145. Review.

[19] Lee LA, Dang CV. Myc target transcriptomes. *Curr Top Microbiol Immunol* 2006, 302, 145-167. *Review.*

[20] Prochownik EV. c-Myc as a therapeutic target in cancer. *Expert Rev Anticancer Ther* 2004, 4, 289-302. *Review.*

[21] Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol Cell Biol* 1999, 19, 1-11. *Review.*

[22] Ehrhardt A, Bartels T, Klocke R, Paul D, Halter R. Increased susceptibility to the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in transgenic mice overexpressing c-myc and epidermal growth factor in alveolar type II cells. *J Cancer Res Clin Oncol* 2003, 129, 71-75.

[23] Chatterji B, Borlak J. Serum proteomics of lung adenocarcinomas induced by targeted overexpression of c-raf in alveolar epithelium identifies candidate biomarkers. *Proteomics* 2007, 7, 3980-3991.

[24] Lehner I, Niehof M, Borlak J. An optimized method for the isolation and identification of membrane proteins. *Electrophoresis* 2003, 24, 1795-1808.

[25] Rabilloud T. Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. *Electrophoresis* 1998, 19, 758-760.

[26] Görg A, Postel W, Weser J, Günther S *et al.* Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. *Electrophoresis* 1987, 8, 122-124.

[27] Rütters H, Zürbig P, Halter R, Borlak J. Towards a lung adenocarcinoma proteome map: studies with SP-C/c-raf transgenic mice. *Proteomics* 2006, 10, 3127-3137.

[28] Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999, 20, 3551-3567.

[29] Chamrad DC, Koerting G, Gobom J, Thiele H *et al.* Interpretation of mass spectrometry data for high-throughput proteomics. *Anal Bioanal Chem* 2003, 376, 1014-1022.

[30] Hood BL, Zhou M, Chan KC, Lucas DA *et al.* Investigation of the mouse serum proteome. *J Proteome Res* 2005, 4, 1561-1568.

[31] Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 2002, 1, 845-867. *Review*. Erratum in: *Mol Cell Proteomics* 2003, 2, 50.

[32] Zeller KI, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV. An integrated database of genes responsive to the Myc oncogenic transcription factor:

identification of direct genomic targets. *Genome Biol* 2003, 4, R69.

[33] Ceciliani F, Giordano A, Spagnolo V. The systemic reaction during inflammation: the acute-phase proteins. *Protein Pept Lett* 2002, 9, 211-223. *Review*.

[34] Kanoh Y, Ohtani N, Mashiko T, Ohtani S *et al.* Levels of alpha 2 macroglobulin can predict bone metastases in prostate cancer. *Anticancer Res* 2001, 21, 551-556.

[35] Misra UK, Pizzo SV. Regulation of cytosolic phospholipase A2 activity in macrophages stimulated with receptor-recognized forms of alpha 2-macroglobulin: role in mitogenesis and cell proliferation. *J Biol Chem* 2002, 277, 4069-4078.

[36] Pepys MB, Herbert J, Hutchinson WL, Tennent GA *et al.* Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis. *Nature* 2002, 417, 254-259.

[37] Korbelik M, Cecic I, Merchant S, Sun J. Acute phase response induction by cancer treatment with photodynamic therapy. *Int J Cancer* 2007, Nov 21

[38] Van Bennekum AM, Wei S, Gamble MV *et al.* Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice. *J Biol Chem* 2001, 276, 1107-1113.

[39] Roberts D, Williams SJ, Cvetkovic D *et al.* Decreased expression of retinol-binding proteins is associated with malignant transformation of the ovarian surface epithelium. *DNA Cell Biol* 2002, 21, 11-19.

[40] Zhang Z, Bast RC Jr, Yu Y, Li J *et al.* Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004, 64, 5882-5890.

[41] Moore LE, Fung ET, McGuire M, Rabkin CC *et al.* Evaluation of apolipoprotein A1 and posttranslationally modified forms of transthyretin as biomarkers for ovarian cancer detection in an independent study population. *Cancer Epidemiol Biomarkers Prev* 2006, 15, 1641-1646.

[42] Maciel CM, Junqueira M, Paschoal ME, Kawamura MT *et al.* Differential proteomic serum pattern of low molecular weight proteins expressed by adenocarcinoma lung cancer patients. *J Exp Ther Oncol* 2005, 5, 31-38.

[43] Feng JT, Liu YK, Song HY, Dai Z *et al.* Heat-shock protein 27: a potential biomarker for hepatocellular carcinoma identified by serum proteome analysis. *Proteomics* 2005, 5, 4581-4588.

[44] Cham BE. Importance of apolipoproteins in lipid metabolism. *Chem Biol Interact* 1978, 20, 263-277. *Review.*

[45] Chen J, Anderson M, Misek DE, Simeone DM, Lubman DM. Characterization of apolipoprotein and apolipoprotein precursors in pancreatic cancer serum samples via two-dimensional liquid chromatography and mass spectrometry. *J Chromatogr A* 2007, 1162, 117-25.

[46] Venanzoni MC, Giunta S, Muraro GB, Storari L *et al.* Apolipoprotein E expression in localized prostate cancers. *Int J Oncol* 2003, 22, 779-786.

[47] Wright LC, Sullivan DR, Muller M, Dyne M *et al.* Elevated apolipoprotein(a) levels in cancer patients. *Int J Cancer* 1989, 43, 241-244.

[48] Han C, Zhang HT, Du L, Liu X *et al.* Serum levels of leptin, insulin, and lipids in relation to breast cancer in china. *Endocrine* 2005, 26, 19-24.

[49] Chang SI, El-Bayoumy K, Sinha I, Trushin N *et al.* 4-(Methylnitrosamino)-I-(3-pyridyl)-1-butanone enhances the expression of apolipoprotein A-I and Clara cell 17-kDa protein in the lung proteomes of rats fed a corn oil diet but not a fish oil diet. *Cancer Epidemiol Biomarkers Prev* 2007, 16, 228-235.

[50] Ishikawa I, Hayama T, Yoshida S, Asaka M *et al.* Proteomic analysis of rat plasma by SELDI-TOF-MS under the condition of prevention of progressive adriamycin nephropathy using oral adsorbent AST-120. *Nephron Physiol* 2006, 103, 125-130.

[51] Hiukka A, Fruchart-Najib J, Leinonen E, Hilden H *et al*. Alterations of lipids and apolipoprotein CIII in very low density lipoprotein subspecies in type 2 diabetes. *Diabetologia* 2005, 48, 1207-1215.

[52] Yokoyama Y, Kuramitsu Y, Takashima M, Iizuka N, *et al.* Protein level of apolipoprotein E increased in human hepatocellular carcinoma. *Int J Oncol* 2006, 28, 625-631.

[53] Li ZG, Zhao L, Liu L, Ding YQ. Monitoring changes of serum protein markers

in metastatic colorectal carcinoma model. *Zhonghua Bing Li Xue Za Zhi* 2007, 36, 48-52.

[54] Grønborg M, Kristiansen TZ, Iwahori A, Chang R *et al*. Biomarker discovery from pancreatic cancer secretome using a differential proteomic approach. *Mol Cell Proteomics* 2006, 5, 157-171.

[55] Grainger DJ, Reckless J, McKilligin E. Apolipoprotein E modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice. *J Immunol* 2004, 173, 6366-6375.

[56] Beecken WD, Engl T, Ringel EM, Camphausen K *et al.* An endogenous inhibitor of angiogenesis derived from a transitional cell carcinoma: clipped beta2-glycoprotein-I. *Ann Surg Oncol* 2006, 13, 1241-1251.

[57] Hough CD, Cho KR, Zonderman AB, Schwartz DR, Morin PJ. Coordinately up-regulated genes in ovarian cancer. *Cancer Res* 2001, 61, 3869-3876.

[58] Iwata K, Nishinaka T, Matsuno K, Yabe-Nishimura C. Increased gene expression of glutathione peroxidase-3 in diabetic mouse heart. *Biol Pharm Bull* 2006, 29, 1042-1045.

[59] Smida J, Holubek V, Oravec C, Thurzo V. The properdin system in tumorous disease. Part VI. Influence of the properdin fraction on the cancerogenic activity of chicken tumour virus B77. *Neoplasma* 1960, 7, 26-30.

[60] Beebe DP, Schreiber RD, Cooper NR. Neutralization of influenza virus by normal human sera: mechanisms involving antibody and complement. *J Immunol* 1983, 130, 1317-1322.

[61] Factor VM, Kiss A, Woitach JT, Wirth PJ, Thorgeirsson SS. Disruption of redox homeostasis in the transforming growth factor-alpha/c-myc transgenic mouse model of accelerated hepatocarcinogenesis. *J Biol Chem* 1998, 273, 15846-15853.

Figure 1a

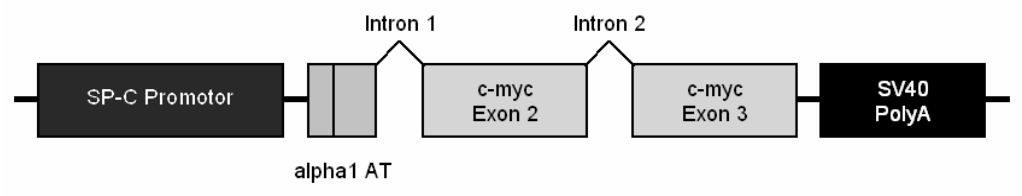

Intron 1
Intron 2
SP-C Promotor
c-myc
Exon 2
c-myc
Exon 3
SV40
PolyA
alpha1 AT

Figure 1b

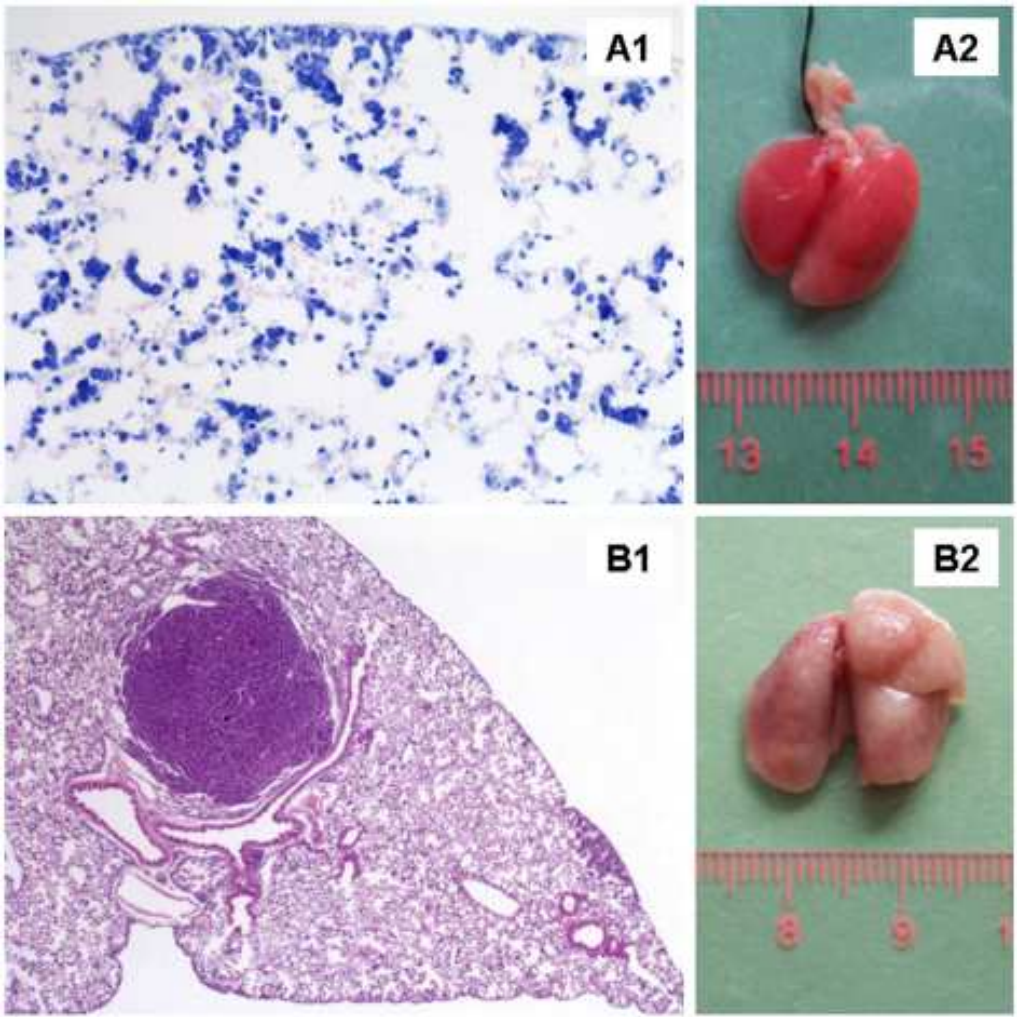


Figure 2a

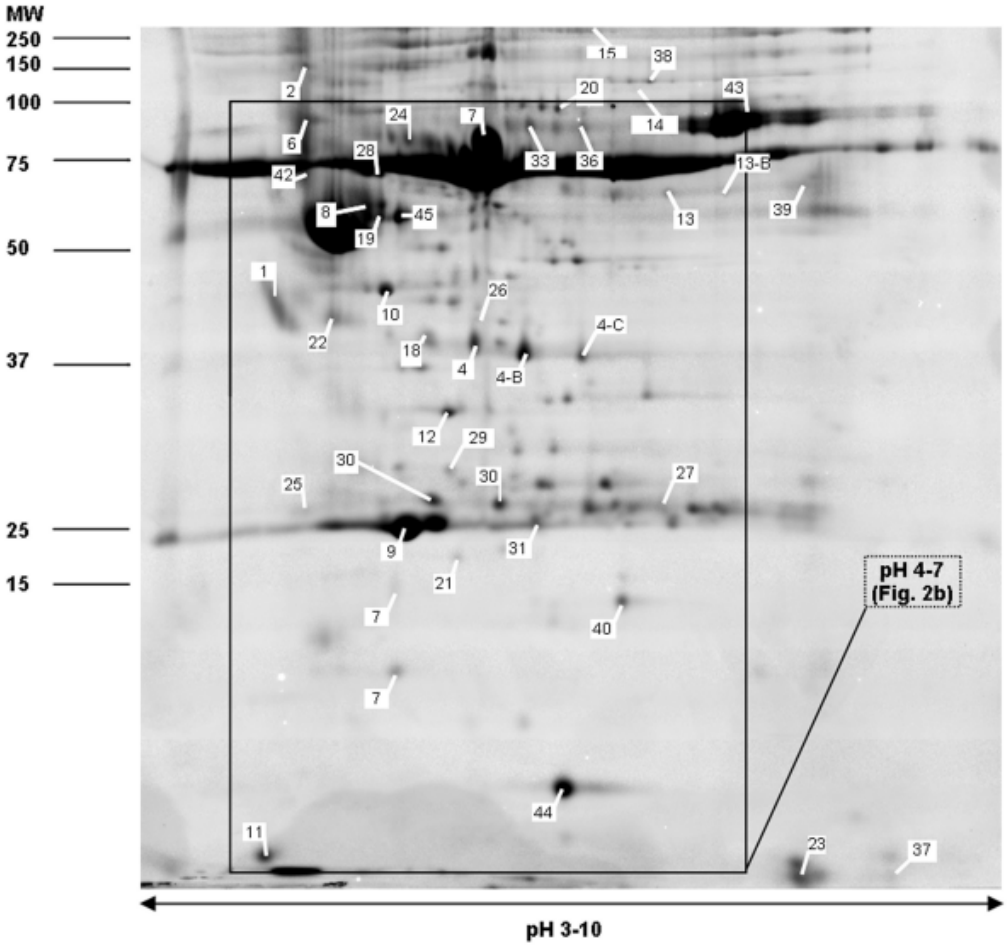


Figure 2b

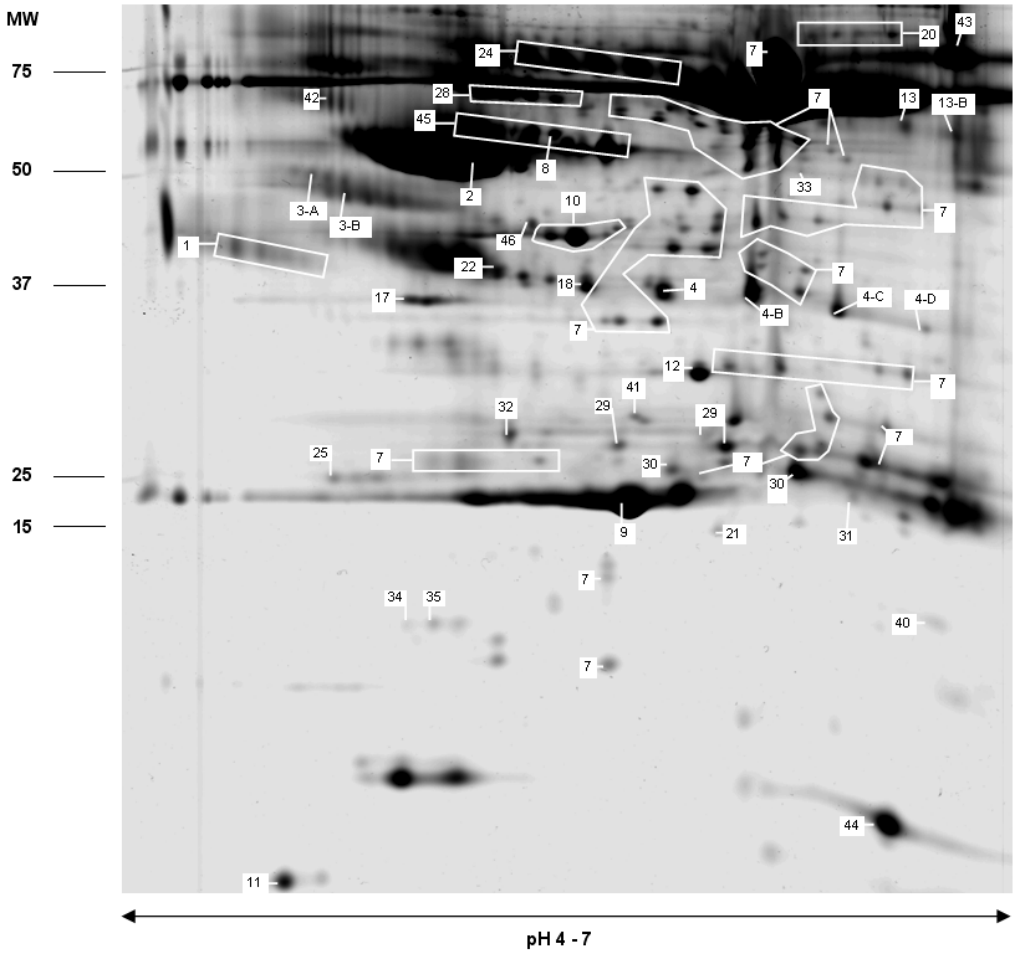


Figure 3a

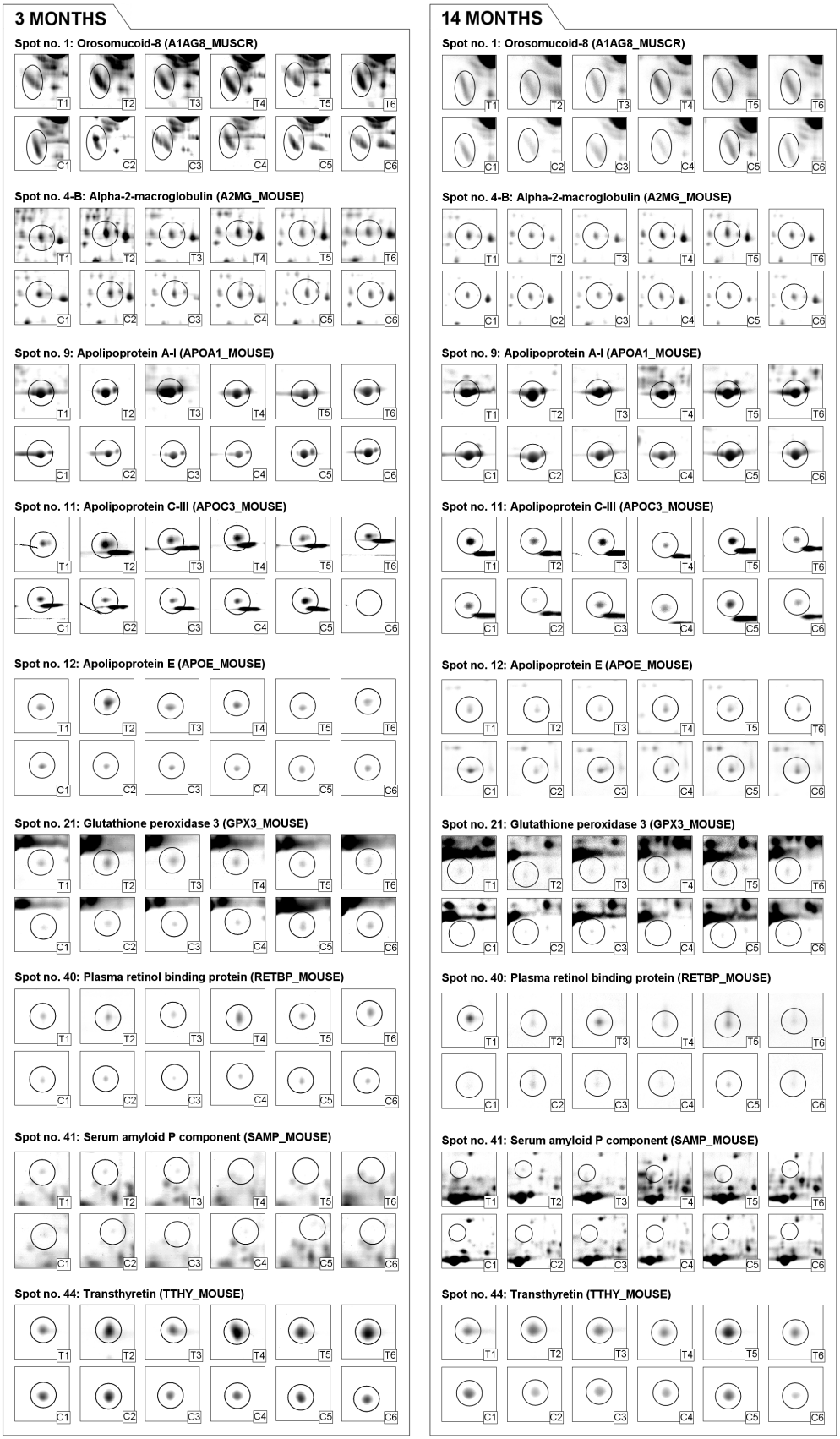

3 MONTHS
14 MONTHS
Spot no. 1: Orosomucoid-8 (A1AG8_MUSCR)
Spot no. 4-B: Alpha-2-macroglobulin (A2MG_MOUSE)
Spot no. 9: Apolipoprotein A-I (APOA1_MOUSE)
Spot no. 11: Apolipoprotein C-III (APOC3_MOUSE)
Spot no. 12: Apolipoprotein E (APOE_MOUSE)
Spot no. 21: Glutathione peroxidase 3 (GPX3_MOUSE)
Spot no. 40: Plasma retinol binding protein (RETBP_MOUSE)
Spot no. 41: Serum amyloid P component (SAMP_MOUSE)
Spot no. 44: Transthyretin (TTHY_MOUSE)
T1
T2
T3
T4
T5
T6
C1
C2
C3
C4
C5
C6

Figure 3b

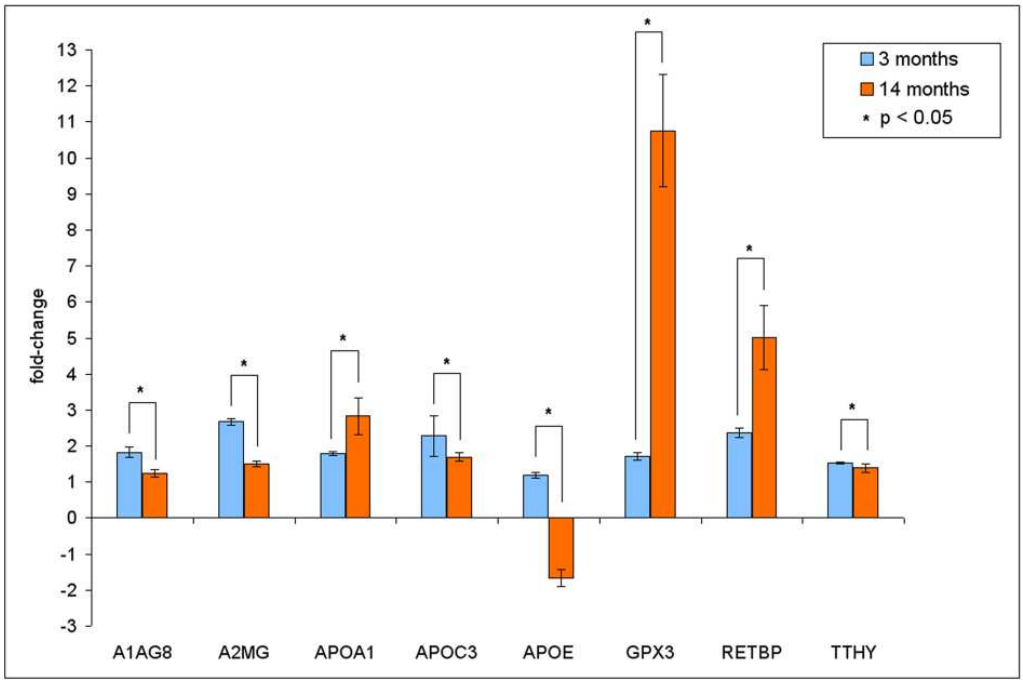

fold-change
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
3 months
14 months
* p < 0.05
A1AG8
A2MG
APOA1
APOC3
APOE
GPX3
RETBP
TTHY

Figure 4

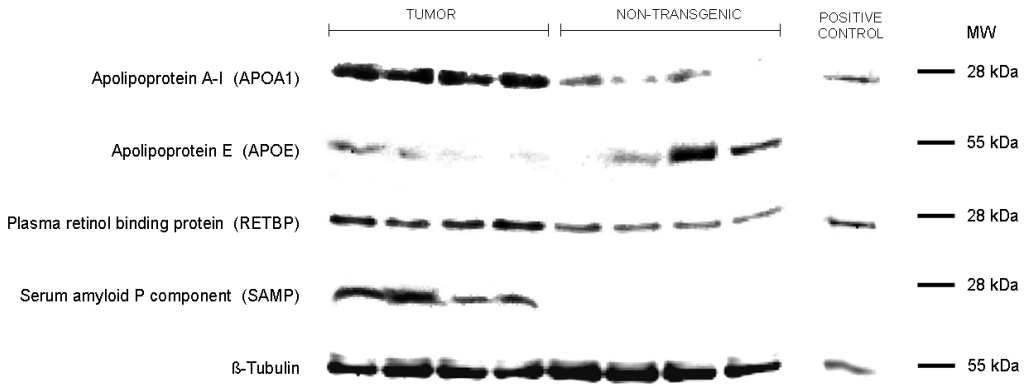

TUMOR
NON-TRANSGENIC
POSITIVE CONTROL
MW
Apolipoprotein A-I (APOA1)
28 kDa
Apolipoprotein E (APOE)
55 kDa
Plasma retinol binding protein (RETBP)
28 kDa
Serum amyloid P component (SAMP)
28 kDa
ß-Tubulin
55 kDa

Table 1

No.	UniProt entry name	UniProt accession no.	Protein name	pI	M, (kDa)	Sequence coverage	No. of matched peptides	Identification
1	A1AG8_MUSCR	P21352	Orosomucoid-8	5.6	23.9	7%	1	PFF
2	A1AT1_MOUSE	P07758	Alpha-1-antitrypsin 1-1	5.4	46.0	26%	10	PMF
3-A, 3-B	A1AT6_MOUSE	P81105	Alpha-1-antitrypsin 1-6	5.3	45.8	34%	11	PMF
						9%	3	PFF
4, 4-B, 4-C, 4-D	A2MG_MOUSE	Q61838	Alpha-2-macroglobulin	6.2	165.9	1%	1	PFF
5	ACTG_MOUSE	P63260	Gamma-actin	5.3	41.8	31%	9	PMF
6	AFAM_MOUSE	O89020	Afamin	5.5	69.4	22%	14	PMF
7	ALBU_MOUSE	P07724	Serum Albumin	5.8	68.7	24%	11	PMF
						2%	1	PFF
8	ANT3_MOUSE	P32261	Antithrombin-III	6.1	52.0	33%	13	PMF
9	APOA1_MOUSE	Q00623	Apolipoprotein A-I	5.6	30.6	40%	15	PMF
						4%	1	PFF
10	APOA4_MOUSE	P06728	Apolipoprotein A-IV	5.4	45.0	64%	21	PMF
11	APOC3_MOUSE	P33622	Apolipoprotein C-III	4.6	11.0	19%	1	PFF
12	APOE_MOUSE	P08226	Apolipoprotein E	5.6	35.9	44%	20	PMF
						4%	1	PFF
13, 13-B	APOH_MOUSE	Q01339	Apolipoprotein H	8.6	38.6	49%	15	PMF
14	CFAB_MOUSE	P04186	Complement factor B	7.2	85.0	18%	12	PMF
15	CFAH_MOUSE	P06909	Complement factor H	6.6	139.1	13%	16	PFF
16	CLUS_MOUSE	Q06890	Clusterin (Apolipoprotein J)	5.5	51.7	12%	4	PMF
17	CO3_MOUSE	P01027	Complement C3	6.4	186.5	8%	10	PMF
18	CRP_MOUSE	P14847	C-reactive protein	5.8	25.4	15%	4	PMF
19	FETUA_MOUSE	P29699	Fetuin-A	6.0	37.3	28%	7	PMF
20	GELS_MOUSE	P13020	Gelsolin	5.8	85.9	31%	19	PMF
21	GPX3_MOUSE	P46412	Glutathione peroxidase 3	8.3	25.3	7%	1	PFF
22	HA10_MOUSE	P01898	H-2 class I histocompatibility antigen, Q10 alpha chain	5.1	37.3	31%	10	PMF
23	HBA_MOUSE	P01942	Hemoglobin subunit alpha	8.0	15.1	37%	5	PMF
24	HEMO_MOUSE	Q91X72	Hemopexin	7.9	51.3	35%	14	PMF
						5%	1	PFF
25	IGJ_MOUSE	P01592	Immunglobulin J chain	4.8	18.0	28%	6	PMF
26	K2C8_MOUSE	P11679	Cytokeratin-8	5.7	54.6	35%	17	PMF
27	KAC_MOUSE	P01837	Immunglobulin kappa chain C region	5.2	11.8	32%	4	PMF
28	KNG1_MOUSE	O08677	Kininogen-1	6.1	73.1	17%	10	PMF
						3%	1	PFF
29	KV3B_MOUSE	P01655	Immunglobulin kappa chain V-III region PC 7132	5.2	12.1	16%	1	PFF
30	KV5L_MOUSE	P01645	Immunglobulin kappa chain V-V region HP 93G7	8.0	12.0	50%	5	PMF
						7%	1	PFF
31	KV6K_MOUSE	P04945	Immunglobulin kappa chain V-VI region NQ2-6.1	9.0	11.7	14%	1	PFF
32	MBL2_MOUSE	P41317	Mannose-binding protein C	5.0	26.0	24%	5	PMF
						5%	1	PFF
33	MUC_MOUSE	P01872	Immunglobulin mu chain C region secreted form	6.6	50.0	2%	1	PFF
34	MUP2_MOUSE	P11589	Major urinary protein 2	5.0	20.7	50%	10	PMF
35	MUP8_MOUSE	P04938	Major urinary proteins 11 and 8	4.9	17.6	72%	10	PMF
36	MYH9_MOUSE	Q8VDD5	Myosin-9	5.5	226.4	5%	8	PMF
37	PLF4_MOUSE	Q9Z126	Platelet factor 4	9.4	11.2	44%	7	PMF
38	PLMN_MOUSE	P20918	Plasminogen	6.2	90.8	25%	21	PMF
39	PROP_MOUSE	P11680	Properdin	8.3	50.3	21%	8	PMF
40	RETBP_MOUSE	Q00724	Plasma retinol-binding protein	5.7	23.2	24%	5	PMF
						8%	1	PFF
41	SAMP_MOUSE	P12246	Serum amyloid P-component	6.0	26.2	8%	1	PFF
42	SPA3K_MOUSE	P07759	Contrapsin	5.1	46.9	32%	14	PMF
43	TRFE_MOUSE	Q921I1	Serotransferrin	6.9	76.7	51%	36	PMF
44	TTHY_MOUSE	P07309	Transthyretin	5.8	15.8	55%	5	PMF
						15%	1	PFF
45	VTDB_MOUSE	P21614	Vitamin D-binding protein	5.4	53.6	30%	9	PMF
						4%	1	PFF
46	ZA2G_MOUSE	Q64726	Zinc-alpha-2-glycoprotein	5.8	35.3	32%	9	PMF
						3%	1	PFF

Table 2a

No.	pH	Protein ID	Tumor (3 months)								Control (3 months)								Ratio (T/C)	p
			T1	T2	T3	T4	T5	T6	mean	%RSD	C1	C2	C3	C4	C5	C6	mean	%RSD		
1	pH 3-10	A1AG8_MUSCR	3856	4255	4040	4232	3453	4196	4005	7.72	2221	1994	1914	2772	2273	1948	2187	14.73	1.8	1.63×10^{-06}
4-B	pH 3-10	A2MG_MOUSE	2064	2195	2205	2278	2128	2146	2169	3.4	794	627	837	990	784	846	813	14.43	2.7	3.62×10^{-10}
9	pH 3-10	APOA1_MOUSE	8228	7526	7911	7777	8011	7826	7880	2.99	4729	4474	4085	4775	4105	4264	4405	6.88	1.8	7.80×10^{-10}
11	pH 3-10	APOC3_MOUSE	1765	1697	1841	1763	1780	795	1606	24.93	815	898	809	758	845	97	704	42.76	2.3	0,00130
12	pH 3-10	APOE_MOUSE	2144	2075	2021	2264	1886	2221	2102	6.6	1714	1782	1730	1916	1765	1827	1789	4.12	1.2	0,00064
21	pH 3-10	GPX3_MOUSE	439	421	403	474	435	418	432	5.67	242	253	247	269	255	251	253	3.61	1.7	1.19×10^{-08}
40	pH 3-10	RETBP_MOUSE	993	995	997	1107	1067	967	1021	5.27	407	417	426	408	494	418	428	7.65	2.4	5.33×10^{-10}
41	pH 3-10	SAMP_MOUSE	178	103	12	4	4	3	50	145.76	9	18	9	14	5	3	10	57.44	5.2	0,20532
44	pH 3-10	TTHY_MOUSE	3568	3654	3629	3693	3690	3542	3629	1.73	2352	2533	2400	2329	2343	2305	2377	3.47	1.5	4.59×10^{-11}

Table 2b

No.	pH	Protein ID	Tumor (14 month's)								Control (14 month's)								Ratio (T/C)	p
			T1	T2	T3	T4	T5	T6	mean	%RSD	C1	C2	C3	C4	C5	C6	mean	%RSD		
1	pH 4-7	A1AG8_MUSCR	1139	1003	1123	998	1217	867	1058	11.88	425	398	401	414	502	387	421	9.91	2.5	3.50×10^{-7}
1	pH 3-10	A1AG8_MUSCR	834	745	769	895	744	867	809	8.07	780	637	545	715	679	552	651	14.20	1.2	6.64×10^{-3}
2	pH 4-7	A1AT1_MOUSE	474	456	494	485	541	391	473	10.39	400	384	482	314	391	327	383	15.65	1.2	1.73×10^{-2}
2	pH 3-10	A1AT1_MOUSE	518	616	549	863	636	663	641	18.99	498	344	475	555	475	562	485	16.26	1.3	2.49×10^{-2}
3-A	pH 4-7	A1AT6_MOUSE	781	848	891	899	884	916	870	5.61	630	686	752	618	812	711	702	10.51	1.2	8.90×10^{-4}
3-B	pH 4-7	A1AT6_MOUSE	450	546	502	446	680	486	518	16.82	349	354	306	359	410	484	377	16.46	1.4	8.85×10^{-3}
3-A/B	pH 3-10	A1AT6_MOUSE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-B	pH 4-7	A2MG_MOUSE	5588	5789	4708	5609	5332	6008	5506	8.19	867	935	766	790	846	910	852	7.74	6.5	2.39×10^{-10}
4-B	pH 3-10	A2MG_MOUSE	6773	6059	6128	6592	5845	6307	6284	5.52	4176	4044	4087	4153	4900	3793	4192	8.89	1.5	1.51×10^{-6}
9	pH 4-7	APOA1_MOUSE	5423	5219	4868	5172	4972	5267	5154	3.93	3490	4036	3362	3379	4004	3313	3597	9.25	1.4	1.93×10^{-6}
9	pH 3-10	APOA1_MOUSE	5952	4324	6205	5549	5869	7721	5937	18.47	1603	1725	2017	2471	2260	2495	2095	18.04	2.8	1.04×10^{-5}
11	pH 4-7	APOC3_MOUSE	3433	3693	3472	3132	3217	3552	3417	6.02	1313	1342	1236	1511	1189	1096	1281	11.17	2.7	1.57×10^{-9}
11	pH 3-10	APOC3_MOUSE	3682	3357	3318	3716	3217	3885	3529	7.57	2372	1630	1749	2522	2131	2018	2070	16.71	1.7	9.73×10^{-6}
12	pH 4-7	APOE_MOUSE	2502	2139	2619	2204	2553	2267	2381	8.48	2478	2312	2823	2908	3116	2935	2762	11.02	0.9	2.85×10^{-2}
12	pH 3-10	APOE_MOUSE	1857	1727	1294	1657	1553	1834	1654	12.65	2993	2207	2688	2876	2598	2978	2723	10.95	0.6	2.95×10^{-5}
13	pH 4-7	APOH_MOUSE	2377	2773	2292	2783	2365	2131	2454	10.85	1525	1330	1774	2073	2020	1947	1778	16.66	1.4	1.97×10^{-3}
13	pH 3-10	APOH_MOUSE	1148	1281	1269	1724	1247	1410	1347	15.08	845	752	809	898	749	683	790	9.75	1.7	9.11×10^{-5}
21	pH 4-7	GPX3_MOUSE	520	339	547	506	358	541	469	20.13	165	124	179	232	229	147	179	24.35	2.6	4.66×10^{-5}
21	pH 3-10	GPX3_MOUSE	189	223	240	233	213	290	231	14.62	19	20	15	20	25	30	22	24.39	10.8	3.45×10^{-8}
39	pH 4-7	PROP_MOUSE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	pH 3-10	PROP_MOUSE	284	311	304	248	240	240	271	12.02	228	156	182	139	217	187	185	18.42	1.5	1.16×10^{-3}
40	pH 4-7	RETBP_MOUSE	1244	1185	1534	1450	1225	1442	1347	10.83	429	466	555	716	521	546	539	18.44	2.5	5.52×10^{-7}
40	pH 3-10	RETBP_MOUSE	407	320	537	402	367	427	410	17.74	66	106	87	63	94	74	82	20.67	5.0	8.00×10^{-7}
41	pH 4-7	SAMP_MOUSE	638	1271	1350	724	523	1496	1000	41.86	0	0	0	0	0	0	0	0	T	1.61×10^{-4}
41	pH 3-10	SAMP_MOUSE	411	967	750	901	501	800	722	30.64	0	0	0	0	0	0	0	0	T	1.19×10^{-5}
44	pH 4-7	TTHY_MOUSE	9952	9049	9204	8992	9869	10721	9631	7.01	8045	7835	6190	9258	5998	5384	7118	20.91	1.4	3.68×10^{-3}
44	pH 3-10	TTHY_MOUSE	9569	10047	8934	8170	8330	9154	9034	7.95	6148	6432	6932	6738	6676	6095	6504	5.18	1.4	1.44×10^{-5}

Table 3

	Protein ID	Protein name	AAH		BAC		APP	E-Box
			1M	12M	3M	14M		
common	A1AT6_MOUSE	Alpha-1-antitrypsin 1-6	↑	↑↑↑	↔	↑	yes	yes
	A2MG_MOUSE	Alpha-2-macroglobulin (A2M)	↓	↑↑	↑↑	↑↑↑	yes	yes
	TTHY_MOUSE	Transthyretin	↑	↓↓↓	↑	↑	yes	no
	PROP_MOUSE	Properdin	-	C	↔	↑	no	no
specific	A1AG8_MUSCR	Orosomucoid-8	↔	↔	↑↑	↑↑	yes	yes
	A1AT1_MOUSE	Alpha-1-antitrypsin 1-1	-	-	-	↑	yes	yes
	APOA1_MOUSE	Apolipoprotein A-I	↔	↔	↑↑	↑↑	no	no
	APOC3_MOUSE	Apolipoprotein C-III	↔	↔	↑↑	↑↑	no	no
	APOE_MOUSE	Apolipoprotein E	↔	↔	↑	↓↓	no	yes
	APOH_MOUSE	Apolipoprotein H	↔	↔	↔	↑↑	no	no
	GPX3_MOUSE	Glutathione peroxidase 3	↔	↔	↑↑	↑↑↑	no	yes
	RETBP_MOUSE	Plasma retinol-binding protein	↔	↔	↑↑	↑↑↑	yes	no
	SAMP_MOUSE	Serum amyloid P-component	-	-	↑↑↑	T	yes	no
	MUP8_MOUSE	Major urinary proteins (MUP)	↑↑↑	↑↑↑	↔	↔	no	no
	VTDB_MOUSE	Vitamin D-binding protein	↓	↑↑	↔	↔	no	no
	S6A11_MOUSE	Sodium- and chloride-dependent GABA transporter 4	-	↑↑↑	↔	-	no	no
	EGFR_MOUSE	Epidermal growth factor receptor (EGFR)	-	C	-	-	no	no

Supplementary Table 1:
A summary of serum proteins from SP-C/c-myc transgenic mice

No.	Swissprot ID	Swissprot Accession no.	Protein aliases	Mascot score (PMF) No. of matched peptides Percentual coverage	Peptide sequences from PMF	Ions score (PFF) No. of matched peptides Percentual coverage	Peptide sequences from PFF	Gene	Subcellular location
1	A1AG8_MUSCR	P21352	Alpha-1-acid glycoprotein 8 [Precursor] AGP 8 Orosomucoid-8 OMD 8	-	-	71 1 7	YEGGVETFAHLIVLR	Orm8 Agp-8 Orm-8	Secreted
2	A1AT1_MOUSE	P37758	Alpha-1-antitrypsin 1-1 [Precursor] Serine protease inhibitor 1-1 Alpha-1 protease inhibitor 1 Alpha-1-antiproteinase AAT	133 13 26	LAQIHFPR TLMSPLGITR TLMSPLGITR + OXIM RLAQIHFPR ELISKFLLHR LDQDTVFALANYILFK KLDQDTVFALANYILFK IFHNGADLSGITEENAPLK DQSPASHEIATHLGDFAISLYR KPFDPENTEEAEFHVDESTTVK	-	-	Serpina1a Dom1 Spi1-1	Secreted
3	A1AT6_MOUSE	P81135	Alpha-1-antitrypsin 1-6 [Precursor] Serine protease inhibitor 1-6 Alpha-1 protease inhibitor 6	123 11 34	LAQIHFPR TLMSPLGITR TLMSPLGITR + OXIM RLAQIHFPR MQHLEQTLSK ELISKFLLHR IFHNGADLSGITEENAPLK NIHYQAEVFSVNFAESEEAK DQSPASHEIATHLGDFAISLYR KPFDPENTEEAEFHVDESTTVK FDHPFLFIFEEHTQSPLFVGK	111, 42, 33 3 9	LAQIHFPR ELISKFLLHR DQSPASHEIATHLGDFAISLYR	Spi1-6 Dom6	Secreted
4	A2MG_MOUSE	Q61838	Alpha-2-macroglobulin [Precursor] Alpha-2-M Pregnancy zone protein	-	-	89 1 1	APFALQVHTLPLHFDK	A2m Pzp	Secreted
5	ACTG_MOUSE	P63710	Actin, cytoplasmic 2 Gamma-actin	131 9 31	IIAPPERK ADFAGDDAPR GYSFTTTAER AVFPSIVGRPR IWHHTFYNELR QEYDESGPSIVHR SYELPDGQVITIGNER VAPEEHPVLLTEAPLNPK DLYANTVLSGGTTMYPGIADR + OXIM	-	-	Actg1 Actg	Cytoplasma
6	AFAM_MOUSE	O89023	Afamin [Precursor] Alpha-albumin Alpha-Alb	131 14 22	FLVNLVK TDFAFRR EIAPAGCYR AAAPTQVLK VMLDYRDR + OXIM HRFLVNLVK RLCPFVNK VLNSINVAVFSK FTESENVCQER VYVMDFLEDCCQR + OXIM AAPQLPMEELVSLSK + OXIM AINYGFLPPFPTLDPEK KAINYGFLPPFPTLDPEEK EACIINAIKDDRPEGLSLR	-	-	Afm	Secreted
7	ALBU_MOUSE	P37724	Serum albumin [Precursor]	124 11 24	CCTLPEDQR HPDYSVSLLLR APQVSTPTLVEAAR RHPDYSVSLLLR LGEYGFQNALIVR DVFLGTFLYEYSR LPCVEDYLSAILNR RPCFSALTVDETYVPK AETFTFHSDICTLPEK AADKDTCFSTEGPNLVTR CCAEANPPACYGTVLAEFQPLVEEPK	95 1 2	LGEYGFQNALIVR	Alb Alb1 Alb-1	Secreted
8	ANT3_MOUSE	P32261	Antithrombin-III [Precursor] ATIII	135 13 33	RVVELSK SLIPNIRVTFK AIIRPELYLR TSDQHFFFAK DIPVNPLCIYR EVALNTIIFMGR + OXIM SSDLVSANIRLFGDK LQPLDFKENFEQSR DIPVNPLCIYRSPGK ATEEDGSEQKVPEATNR AILEVNEEGSEAAASTSVVITGR SQLPGIVAGGRDDLYVSDAFHK	-	-	Serpinc1 At3	Secreted extracellular space
9	APOA1_MOUSE	Q00623	Apolipoprotein A-I [Precursor] Apo-AI ApoA-I	96 15 43	LQELQGR EDVELYR AIPALEDLR LSPVAEEFR QKLQELQGR VKEDVELVR VQPYLDEFQK TQLAPHSEQMR TQLAPHSEQMR + OXIM LSPVAEEFRDR SNPTLNEYHTR VAPLGAELQESAR QEMNKDLEEVK VKDFAIVYVDAVK LQELQGRLSPVAEEFR	84 1 4	VAPLGAELQESAR	Apoa1	Secreted
10	APOA4_MOUSE	P36728	Apolipoprotein A-IV [Precursor] Apo-AIV ApoA-IV	235 21 64	EAVEQFQK LQLTPYQR ALVQQLEQFR SLAPLTVQVQEK TVEPAGEMPNK + OXIM QLEQQVEEFR NLAPLVEDVQSK TVEPAGEMPNK + 2 OXIM NMEELKGHLTPR + OXIM ATIDQNLEDLRR LGDASTVADQVHNK QLEQQVEEFRR LNHQMEGLAFQMK + 2 OXIM TDVTQQLTLFDDK LNHQMEGLAFQMKK + 2 OXIM LVPFVVQLSGHLAKETER QQLGPNSGEVEESHLSFLEK MMPHANKVTQTFGENMQK + 3 OXIM LQEHLSPVAYDLQDQIRTQTQEMK + OXIM GSPDQPQALPLPEQAQEQAQEQAQEQVQPKPLES KGSPDQPQALPLPEQAQEQAQEQAQEQVQPKPLES	-	-	Apoa4	Secreted
11	APOC3_MOUSE	P33622	Apolipoprotein C-III [Precursor] Apo-CIII ApoC-III	-	-	128 1 19	TVQDALSSVQESDIAVVAR	Apoc3	Secreted
12	APOE_MOUSE	P38226	Apolipoprotein E [Precursor] Apo-E	138 23 44	EVQAAQAR FWDYLR DRLEEVR EHMEEVR + OXIM LGPLVEQGR LEEVGNQAR QWAAVLMEK DRAQAFGDR LQAEIFQAR MEEQTQQIR MEEQTQQIR + OXIM GRLEEVGNQAR TAHLGAGAAQPLR SKMEEQTQQIR SKMEEQTQQIR + OXIM GWVEPYEDMHR GWVEPYEDMHR + OXIM ELEEQLGPVAEETR NEVHTMLGQSTEEIR NEVHTMLGQSTEEIR + OXIM	81 1 4	ELEEQLGPVAEETR	Apoe	Secreted
13	APOH_MOUSE	Q31339	Beta-2-glycoprotein 1 [Precursor] Beta-2-glycoprotein I Apolipoprotein H Apo-H B2GPI Beta(2)GPI Activated protein C-binding protein APC inhibitor	153 15 49	IHFYCK ATVLYQGMR ATVLYQGMR + OXIM ITCPPPVPK WSPDPACAR DGTEIPSCFK CSYTVEAHCR TGTWSFLPTCR VCPFAGILENGAVR IQEQFRNGMMHGDK FTCPLTGMAVPHTLR FTCPLTGMAVPHTLR + OXIM ICPKPGDLPFATVVPLK TSYDPQEQVVSCKPGYVSR CPFPPRPENGYVNYPAKPVDLYK	-	-	Apoh B2gp1	Secreted

14	CFAB_MOUSE	P34186	Complement factor B [Precursor] EC 3.4.21.47 C3/C5 convertase	133 12 18	CLTNLIEK VASYQVIPPR QQLVPSYAR ALLDIGRDPK VKDASEVVTPR YGLLTYATVPK ALRLPQTATCK DLEIEEVLVPHPK YGLLTYATVPKVLVR VGSQVRLEDVTYHCSR YGQTLRPICLPCTEGTTR FIQVGVISWGVGCAVDVCRDQR	-	-	Cfb Bf H2-Bf	Secreted
15	CFAH_MOUSE	P36939	Complement factor H [Precursor] Protein beta-1-H	116 16 13	TGEQVTFR ARDSPPFR HGVYPKER YLHQDRVR CVEILCTPPR IIHRSDDEIR CTPTGWIPYPR CLPVTELENGR SYRTGEQVTFR CVATDQLEKCR VLKSTGIEAIKPK VGDLLEFSCHSGHR TDCDVLPYVKNAIR KKPCGHPGDTPFGSFR SCDAMPVESTSITKITR + OXPM YRVGDLLEFSCHSGHR	-	-	Cfh Hf1	Secreted
16	CLUS_MOUSE	Q06890	Clusterin [Precursor] Sulfated glycoprotein 2 SGP-2 Clustrin Apolipoprotein J Apo-J	68 4 12	RPHFLVPK ASGHDTLFQDR ELHDPHVYSPIGFPHK VYLRVSTVTTHSSDSVPSR	-	-	Clu Apoj Msgp-2	Secreted
17	CO3_MOUSE	P01027	Complement C3 [Precursor] Synonym HSE-MSF	67 13 8	VVLNEQR RQEALELIK DFDSYRPVVR EADVSLTAFVLIALQEAR LLDDFDEYTMTIQQVIK + OXPM ILQGSPVVQMAEDAVDGER VDVPAADLSDQVPDTDSETR ILQGSPVVQMAEDAVDGER + OXPM VFSLAANLIAIDSMVLCGAYK QPSSAVAAFNIRRPDSTWLTAYVVK	-	-	C3	Secreted
18	CRP_MOUSE	P14847	C-reactive protein [Precursor]	62 4 15	TFGHEDMIFK TFGHEDMFFK.K NSNDILIFVNK LLNVCLIMSFSR + OXPM	-	-	Crp Ptx1	Secreted
19	FETUA_MOUSE	P29699	Alpha-2-HS-glycoprotein [Precursor] Fetuin-A Countertrypin	72 7 28	QVLHQIDKVK GLSDHRTVHDLR VGQPQAAGPVSPMCPGR + OXPM AHLMNHLGGEEVSVACK + OXPM QLTEHAVEGDCDFHILK HAFSPVASVESASGETHSPK QLTEHAVEGDCDFHILKQDGQFR	-	-	Ahsg Fetua	Secreted
20	GELS_MOUSE	P13020	Gelsolin [Precursor] Actin-depolymerizing factor ADF Brevin	176 19 31	MDAHPPR + OXPM LFACSNR RTPITVVR EPGLQMVR QFFKNVAR DGGQTAPRSIR YIETDPANR AGKEPGLQMVR HVYPNEVVVQR SEDCFILDHGR EVQGFESSTFSGYFK TPSAAYLWVGAGASEAEK QTQVSVLPEGGETPLFK DPDQTDGPGLGYLSSHMAIVER SQHVQYEGSEPDAFVKELAGGK VSNGAGSMSVSLVADENPFAQGALR VSNGACSMSYSLVADENPFAQGALR + OXPM FDLVPVPPNLYGDFFTGDAYVVLK VPVDPATYGQFYGGDSYIILYNYR	-	-	Gsn Gsb	Secreted (Isoform 1) Cytoplasma (Isoform 2)
21	GPX3_MOUSE	P46412	Glutathione peroxidase 3 [Precursor] EC 1.11.1.9 GSHPx-3 GPx-3 Plasma glutathione peroxidase GSHPx-P	-	-	40 1 7	YVRPGGGFVPNFQLFEK	Gpx3	Secreted

22	HA13_MOUSE	P01898	H-2 class I histocompatibility antigen, Q13 alpha chain [Precursor]	153 13 31	FDSDAETPR GYLQYAYDGR GNEQSFHVSLR VKEQAGAAEYYR KWEQAGAAEYYR AKGNEQSFHVSLR AYLEAECVEWLLR FIYGYVDDTQFVR YFETSVSRPGLGEPR THVTHHPGSEGDVTLR	-	-	H2-Q13	Membrane
23	HBA_MOUSE	P01942	Hemoglobin subunit alpha Hemoglobin alpha chain Alpha-globin	74 5 37	LRVDPVNFK IGGHGAEYGAEALER TYFPHFDVSHGSAQVK TVFPHFDVSHGSAQVKGHGK IGGHGAEYGAEALERMFASFPTTK + OXPM	-	-	Hba Hba-a1	Extracellular
24	HEMO_MOUSE	Q91X72	Hemopexin [Precursor]	126 14 35	GPDSVFLIK YYCFQGNK DYFVSCPGR WPWDFATR NPETSYDAAFR FNPYTQEVPPR KWFWDFATR WKNPETSYDAAFR GATYAFTGSHYWR CSPDPGLTALLSDHR GECQDEGILFFQGNR SLPQPQKVTNSLIGCSQ ELGSPPGISLETIDAAFSCPGSSR LFQEEPGSPVPPDAAVECHR	113 1 5	ELGSPPGISLETIDAAFSCPGSSR	Hpx Hpxn	Secreted
25	IGJ_MOUSE	P01592	Ig J chain	89 6 28	CVTTMVPLR + OXPM NFVVHLSDVCK.K NFVVHLSDVCKK.K IIPSTEDPIEDIVER CVTTMVPLRYHGETK + OXPM IIPSTEDPIEDIVERNIR	-	-	Igj	
26	K2CB_MOUSE	P11679	Keratin, type II cytoskeletal 8 Cytokeratin-8 CK-8 Keratin-8 K8 Cytokeratin endo A	135 17 35	HGDDLRR SFTSGPGAR FLEQQNK VSSSSSFR QIHEEEIR AQYEDIANR FASFIDKVR SYKMSTSGPR YEELQTLAGK TKTEIEMNR + OXPM AQYEDIANRSR SLDMDGIAEVR + OXPM ALSEAAADAEQR SRAEAETMYQIK + OXPM LESGMQNMSIHTK + 2 OXPM DVDEAYMNKVELESR + OXPM ELQSQISDTSVVLSMDNSR + OXPM	-	-	Krt8 Krt2-8	
27	KAC_MOUSE	P01837	Ig kappa chain C region	73 4 32	SFNRNEC VKIDGSER HNSYTCEATHK HNSYTCEATHKTSTSPIVK	-	-		
28	KNG1_MOUSE	Q3B677	Kininogen-1 [Precursor]	75 13 17	VVIETAR RPPGFSPFR SGNQVMLHR SQNQVMLHR + OXPM ENEFFVTQTCK DAEEAATGECTATVGK FPSLHGDCVALPHGDDGECR HLGQSLDCNANYYMRPWENK EVLGHSIAQLHAENDHPFYVK HLGQSLDCNANYYMRPWENK + OXPM	122 1 3	FPSLHGDCVALPHGDDGECR	Kng1 Kng	Secreted Extracellular
29	KV3B_MOUSE	P01655	Ig kappa chain V-III region PC 7132	-	-	139 1 16	DVLTQSPASLAVSLGQR		
30	KVSL_MOUSE	P01645	Ig kappa chain V-V region HP 93G7	61 5 50	LHSGVPSR LLIYYTSR DIQMTQTTSSLSASLGDR DIQMTQTTSSLSASLGDR + OXPM ASQDISNYLIWYQQKPDGTVK	53 1 7	LLIYYTSR		
31	KV6K_MOUSE	P34945	Ig kappa chain V-VI region NQ2-6.1	-	-	115 1 14	LLIYDTSNLASGVPVR		

32	MBL2_MOUSE	P41317	Mannose-binding protein C [Precursor] MBP-C Mannan-binding protein RA-reactive factor P28A subunit RARF/P28A	62 5 24	IMSLDRVK + OXIM DAVLGITDYR VEGSFEDLTGNR ALCSEFQGSVATPR AEFDTSEDSEIAALR	43 1 5	ALCSEFQGSVATPR	Mbl2	Rough endoplasmic reticulum membrane; Peripheral membrane protein; Lumenal side. Golgi apparatus membrane; Peripheral membrane protein; Lumenal side
33	MUC_MOUSE	P31872	Ig mu chain C region secreted form	-	-	54 1 2	HPPAVYLLPPAR	Igh-6	Secreted
34	MUP2_MOUSE	P11589	Major urinary protein 2 [Precursor] MUP 2	124 13 53	IEDNGNFR EKIEDNGNFR ENIIDLSNAHR LFLEQNVLEK FAKLCEEHGILR DGETFQLMGLYGR DGETFQLMGLYGR + OXIM INGEWVHTILASDKR AGEYSVTYDGFNTFTIPK IEDNGNFRLFLEQNVLEK	-	-	Mup2	Secreted
35	MUP8_MOUSE	P34938	Major urinary proteins 11 and 8 [Fragment] MUP11 and MUP8	139 13 72	IEDNGNFR EKIEDNGNFR ENIIDLSNAHR FAQLCEEHGILR DGETFQLMGLYGR DGETFQLMGLYGR + OXIM INGEWVHTILASDKR TDYDNFLMAHLINEK + OXIM LFLEQNVLENSLVLK AGEYSVTYDGFNTFTIPK	-	-	Mup8 Mup11	Secreted
36	MYH9_MOUSE	Q8VDD5	Myosin-9 Myosin heavy chain 9 Myosin heavy chain, nonmuscle IIa Nonmuscle myosin heavy chain IIa NMHMHC II-a NMMHC-IIA Cellular myosin heavy chain, type A Nonmuscle myosin heavy chain-A NMMHC-A	54 8 5	VLQRNCAAYLR VDYKADEVLMK QIATLHAQVTDMK AQQAADKYLVYDK LMATLRNTNPNFVR + OXIM VIQYLAHVASSHKSK YRFLSNGHVTIPGQQDK KANLQIDQINTDLNLER	-	-	Myh9	
37	PLF4_MOUSE	Q9Z126	Platelet factor 4 [Precursor] PF-4 CXCL4	122 7 44	TISGIHLK HITSLEVIK HITSLEVIKAGR HCAVPQLATLK ICLDPQAPLVK AGRHCAVPQLATLK HCAVPQLATLKNGR	-	-	Pf4	Secreted
38	PLMN_MOUSE	P20918	Plasminogen [Precursor] EC 3.4.21.7	173 21 25	SFQYHSK LILEPNNR SSRPEFYK DVILFEKR VVSEQTPHR WEYCDIPR MRDVILFEK + OXIM WEYCNLKR CEGETDFVCR HSFTPQTNPR VILGAHEEYIR LKEAQLPVIEINK TGIGNGYRGTMSR + OXIM VCNRVEYLNVR VVSEQTPHRNHIR GTVSVTVSGKTCQR LILEPNNRDIALLK VIPACLPSPRYYVVADR + OXIM TAVTAAGEPCQGWAAQEPHR SFQYHSKEQQCVIMAENSK + OXIM QLAAGGYSDCLAKCEGETDFVCR	-	-	Plg	Secreted
39	PROP_MOUSE	P11683	Properdin	89 8 21	TCDHPAPR QRLCTPLPK HGGPFCAGDATR MSINCEGTPQQQSR + OXIM LRMSIRCEGTPQQQSR + OXIM HGGPFCAGDATRNQMCNK + OXIM CGGHCPGEAQGSQACEGEQK SCSAPAPSHQPPGKPCSGPAYEHK	-	-	Cfp Pfc	Secreted

40	RETBP_MOUSE	Q03724	Plasma retinol-binding protein [Precursor] PRBP RBP	63 5 34	DPNGLSPETR VVGGVASFLQR QRQEELCLER LQNLDGTCADSYSFVFSR KDPEGLFLQDNIIAEFSVDEK	98 1 8	LQNLDGTCADSYSFVFSR	Rbp4	Secreted
41	SAMP_MOUSE	P12246	Serum amyloid P-component [Precursor] SAP	-	-	139 1 8	APPSIVLGQEQDNYGGGFQR	Apcs Ptx2 Sap	Secreted
42	SPA3K_MOUSE	P37759	Serine protease inhibitor A3K [Precursor] Serpin A3K Contrapsin SPI-2	135 14 32	VPMMKMK MKLLTTR + OXIM FSASIIVR LSVSQVVHK KLSTSQVVHK GKTMEELEGLK + OXIM MQQVEASLQPETLR MQQVEASLQPETLR + OXIM MQQVEASLQPETLRK + OXIM HFRDEELSCSVLELK AVLDVAETGTEAAAATGVIGGIR ALYQTEATTADFQQPTEAK AVLDVAETGTEAAAATGVIGGIRK ISFDPQDTFESEFVLDEKR	-	-	Serpina3k Mcm2 Spi2	Secreted
43	TRFE_MOUSE	Q92111	Serotransferrin [Precursor] Transferrin Siderophilin Beta-1-metal-binding globulin	354 36 51	DSAFGLLR SCHTGLGR VAQEHFGK SCHTGVDR IPSHAVVAR TVLPPDGPR GYYAVAVVK LPEGTTPEK KSCHTGLGR VPPRMDYR + OXIM DLLFRDDTK WCALSHLER CGFRDHMVK LLEACTFHK.H EEYNGYTGAFR HTTIFEVLPEK ASDTSITWNNLK WCAVSEHENTK CLVEKGDVAFVK LYLGHNYVTAIR SKDFQLFSSPLGK GTDFQLNQLEGKK DLLFKDSAFGLLR DQYELLCLDNTR QEDFELLCPDGTR YLGAEYMQSVGNMR + 2 OXIM KPVDQYEDCYLAR YLGAEYMQSVGNMRK + 2 OXIM ADRDQYELLCLDNTR NLKQEDFELLCPDGTR NQQEGVCPEGSDNSPVK CAPNNKEEYNGYTGAFR DFASCHLAQAPNHVVVSR HQFVLDNTEGKNPAEWAK KPVKDFASCHLAQAPNHVVVSR AVLTSQETLFGGSDCTGNFCLFK	-	-	Tf Trf	Secreted
44	TTHY_MOUSE	P37339	Transthyretin [Precursor] Prealbumin	79 5 55	FVEGVYR TSEGSWEPFASGK TAESGELHGLTTDEK TLGISPFHEFADVVFTANDSGHR HYTIAALLSPYSYSTTAVVSNPQN	167 1 15	TLGISPFHEFADVVFTANDSGHR	Ttr	Secreted
45	VTDB_MOUSE	P21614	Vitamin D-binding protein [Precursor] DBP Group-specific component Gc-globulin VDB	131 9 33	SLSILYSR HLSLLTTMSNR RTQVPEVFLSK SCESDAPFPVHPGTPECCTK DLCGQSTTQAMDQVTFELSR DLCGQSTTQAMDQVTFELSR + OXIM VPTANLENYLPLAEDFTEILSR EVVSLTEECCAEGADPTCYDTR LCMAALSHQPQEPPTYVEPTNDEICEAFR	117 1 4	VPTANLENYLPLAEDFTEILSR	Gc	Secreted
46	ZA2G_MOUSE	Q64726	Zinc-alpha-2-glycoprotein [Precursor] Zn-alpha-2-glycoprotein Zn-alpha-2-GP	125 9 32	LAFEPER KLAFEPER IDPPTVTITSR CLAYGFYPQR AREEFLYTLK GFSQSLSVQWDR YAYDGEDFIEFNK AYLEECPEMLKLR + OXIM FQATAFLNDQAFFHYNSNGK	39 1 3	CLAYGFYPQR	Azgp1	Secreted

Publikation III:

Search for novel serum biomarkers of dysplasia in a transgenic mouse model of lung cancer

Running title: Serum proteomics of lung dysplasia

Submitted for publication, 2009

Bijon Chatterji [†‡], Jürgen Borlak [†‡]

[†] Department of Molecular Medicine and Medical Biotechnology, Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM), Hannover, Germany
[‡] Center for Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany

Corresponding author:

Prof. Dr. J. Borlak
Department of Molecular Medicine and Medical Biotechnology
Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM)
Nikolai-Fuchs-Strasse 1
D-30625 Hannover, Germany
Phone: +49-511-5350-559
Fax: +49-511-5350-573
E-mail: borlak@item.fraunhofer.de

Keywords: EGF / Serum / Proteomics / Dysplasia / Lung Cancer

Abbreviations:

2-DE, two-dimensional gel-electrophoresis; **A2M**, α-2-macroglobulin; **AAH,** atypical adenomatous hyperplasia; **AAT**, α-1-antitrypsin; **ApoA1,** apolipoprotein A-I; **ApoA4,** apolipoprotein A-IV; **ApoC3**, apolipoprotein C-III; **ApoE**, apolipoprotein E; **ApoH,** apolipoprotein H; **ApoM,** apolipoprotein M; **APP,** acute phase protein; **a-raf,** serine-threonine kinase of the Raf family; **BAC,** bronchiolo-alveolar carcinoma; **CCB,** colloidal Coomassie blue; **c-myc,** v-myc avian myelocytomatosis viral oncogene homolog; **c-raf,** serine-threonine kinase of the Raf family; **EGF,** epidermal growth factor; **EGFR,** epidermal growth factor receptor; **ERK,** extracellular signal-regulated kinase; **GPX3**, glutathione peroxidase 3; **GSN,** gelsolin; **IEF,** isoelectric focusing; **IPG,** immobilized pH gradient; **MALDI-TOF,** matrix-assisted laser desorption/ionization-time of flight; **MAPK,** mitogen-activated protein kinase; **MUP**, major urinary protein; **NSCLC,** non-small cell lung carcinoma; **PLG,** Plasminogen; **RETBP**, plasma retinol binding protein; **RTK,** receptor tyrosine kinase; **SAP**, serum amyloid P component; **SP-C,** surfactant protein-C; **TTHY,** transthyretin; **VPS28,** vacuolar protein sorting-associated protein 28 homologue; **VTDB,** vitamin D binding protein

Abstract

Aberrant signaling of the epidermal growth factor (EGF) receptor tyrosine kinase is frequently observed in lung cancer. We therefore developed a transgenic mouse model with targeted overexpression of EGF to respiratory epithelium. As evidenced by histopathology, disease bearing mice developed dysplasia and subsequently bronchiolo-alveolar carcinomas. Based on two-dimensional gel-electrophoresis we compared the serum proteomes of diseased mice with wildtype animals. Three pH ranges, 3-10, 4-7 and 5-8 were applied for isoelectric focusing, followed by SDS-PAGE. In total, sixty eight unique serum proteins were identified by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry, of which thirteen proteins were statistically significantly regulated ($p<0.05$). Notably, while EGF and amphiregulin were exclusively expressed in disease bearing animals, expression of α-2-macroglobulin, apolipoprotein A-I, fetuin B, gelsolin, vacuolar protein sorting associated protein 28 homologue and vitamin D binding protein was specifically increased; in contrast, expression of apolipoprotein A-IV, apolipoprotein M, apolipoprotein H, a-raf, glutathione peroxidase 3, plasminogen and transthyretin was repressed in transgenic mice with dysplasia. Furthermore, apolipoprotein A-I, A-IV and ApoM were significantly downregulated while α-2-macroglobulin and glutathione peroxidase 3 were upregulated ($p<0.05$) in lung tumour bearing mice. Regulation of selected proteins was also confirmed by Western blotting. Finally, we compared the serum proteome of three different transgenic lung cancer disease models and identified α-2-macroglobulin and transthyretin as commonly regulated. Taken collectively, we identified several serum biomarker candidates that enabled detection of dysplasia prior to malignant transformation.

1 Introduction

The epidermal growth factor (EGF) is a ligand of the EGF receptor tyrosine kinase (RTK), and this protein displays mitogenic activity *in vivo* and *in vitro*. In many tumours EGFR is overexpressed to result in aberrant signaling [1]. Next to EGF, amphiregulin, epiregulin and TGFα bind to and activate this membrane-bound RTK. Upon ligand activation, the EGFR becomes internalized whereby the intracellular domain undergoes multiple rounds of phosphorylation [2]. Recently, we and others identified a soluble form of the EGFR in serum of lung tumour bearing mice [3, 4]. While overexpression of constitutively activated EGFR resulted in dysregulation of this RTK it provided an autocrine loop to promote mitogenic signaling thereby fostering cell cycle progression [5]. An oncogenic signaling of EGFR was already demonstrated for hepatocellular carcinomas [6-8]. In this regard it is of considerable importance that EGFR and other RTK connect to other signaling pathways, such as the MAPK/ERK signal transduction pathway to promote malignant transformation [9]. There is evidence for selective phosphorylations of individual tyrosine residues to enable specific signaling cascades. For instance, phosphorylation of the EGFR tyrosine residue Tyr992 connects EGF to the MAPK pathway [10-12]. To better understand aberrant EGF signaling in malignancies our laboratory generated a transgenic mouse model where overexpression of the secretable EGF (IgEGF) was achieved by use of a gene construct that contained regulatory sequences of the surfactant protein C (SP-C) promoter. Activation of this promoter is restricted to alveolar epithelium and transgenic mice developed dysplasia in lung [13, 14]. Notably, while atypical adenomatous hyperplasias (AAH) represent a preneoplastic state of carcinomas [15], there is a link between AAH and lung cancer. Indeed, dysplasia display nuclear atypia, which, however, has not yet progressed to the point of malignant transformation and invasive growth [16]. Dysplastic cells are facultative cancers and thus represent a developmental stage of cellular dedifferentiation with high risk for malignant transformation. Because exaggerated activity of the EGF RTK is considered to be a disease causing mechanism of lung cancer, we wished to identify serum biomarkers of disease that would allow prediction and monitoring

of patients at risk for developing lung cancer. Likely identifying individuals at risk of developing cancer would reduce significantly the morbidity associated with lung cancer [17]. Here we report our efforts to study the serum proteome of SP-C/IgEGF transgenic mice and suggest candidate biomarkers for AAH.

2 Materials and methods

2.1 Animal care

Transgenic disease bearing mice and wildtype age matched mice without neoplastic formation of the lung that served as controls were bred in the hemizygous CD2F1 and kept in the C57BL/6 as described previously [13]. Animals were housed in Makrolon type III cages. Drinking water and food (V1124-000, SSNIFF, Holland) was given *ad libitum*. Temperature and relative humidity were 22 ± 2° C and 40-70%, respectively. Furthermore, a 12-h day and night cycle was used.

2.2 Histopathology

Tissues were fixed in 4% buffered formaldehyde in PBS for approximately 20 h, dehydrated and embedded in paraffin (Roti-Plast™, Roth). Tissue captions were obtained with a microtome and stained with hematoxylin and eosin according to standard protocols. Neoplasias of the lung were classified according to standards of the IARC - WHO (2004).

2.3 Preparation of serum samples

Blood serum of transgenic SP-C/IgEGF mice with dysplasia of the lung (n=6, aged 12-14 months), blood serum of transgenic SP-C/IgEGF lung tumor bearing mice (n=4, aged 18-20 months) and blood serum of wildtype mice (n=6, aged 14 months and n=6, aged 18-20 months) were studied. For serum protein

identification, mice were sacrificed with CO_2 and blood from these two groups was taken from the *vena cava*. After clotting for 15 min at room temperature, the blood was centrifuged at 6000 rpm for 10 min. Hemolysis was not observed. The resultant supernatants were removed, frozen immediately in liquid nitrogen and stored at -80° C until further analysis. The protein concentration in serum determined by the Bradford test ranged from 80-90 µg/µL.

2.4 Two-dimensional gel-electrophoresis

2.4.1 First dimension

Serum proteins were separated without any pre-treatment by isoelectric focusing (IEF). We used precast immobilized pH gradient (IPG) strips of pH 3-10 (non-linear gradient; 170x3x0.5 mm, BioRad) and overlapping ranges of pH 4-7, pH 5-8 (both linear gradient; 170x3x0.5 mm, BioRad). Each sample was analyzed in duplicate. 1 mg was diluted in a lysis buffer containing 2 mol/L thiourea, 5 mol/L urea, 40 mmol/L Tris, 4% CHAPS, 0.5% BioLyte 3-10 (BioRad), 100 mmol/L DTT resulting in a total volume of 350 µL per strip. Focused IPG strips were rehydrated at 50 V for 12 h. IEF was performed at 20°C with a maximum voltage of 10 kV and a maximum current of 50 µA per strip. After IEF, IPG strips were equilibrated in 10 mL reducing buffer (2% DTT in 10 mL equilibration buffer containing 6 mol/L urea, 30% glycerin, 2% SDS, 0.05 mol/L Tris-HCl, pH 8.8 and 0.5% bromophenol blue) for 15 min, followed by equilibration in 10 mL alkylation buffer (4% iodoacetamide and 0.5% bromophenol blue in 10 mL equilibration buffer) for 15 min.

2.4.2 Second dimension

SDS-PAGE was performed in a Protean-plus Dodeca™ Cell (BioRad) using self-cast polyacrylamide gels (200 x 205 x 1.5 mm; 12%T). Gels were run in parallel in 0.025 mol/L Tris/ 0.192 mol/L glycine/ 0.1% SDS cooled externally to 10° C with a constant voltage of 70 V for approximately 16 h, followed by 200 V until the

bromophenol blue dye reached the bottom of the gel. Unstained protein standards (BioRad) were used for calibration of M_r and p*I*.

2.5 Staining

2-D gels were fixed overnight in 500 mL 30% ethanol/ 2% phosphoric acid and washed twice for 30 min each in 500 mL 2% phosphoric acid. Equilibration was done in 500 mL 2% phosphoric acid/ 18% ethanol/ 15% ammonium sulfate for 30 min thereafter. Colloidal Coomassie blue (CCB) staining of proteins was performed by addition of 5 mL staining solution (2% CBB G250, Roth) to 500 mL of equilibration solution. Gels were stained for 72 h and washed once with 500 mL deionized water for 5 min thereafter.

2.6 Imaging

Gels were scanned as TIFF images with the 16-bit Expression 10000 XL™ (Epson) operating in transmitted light mode. PDQuest software (version 8.0.1.55, BioRad) was used for detection of spots, quantification and comparison of 2-D protein profiles. Background and vertical streaks were removed from each gel image; spots were digitized by Gaussian fit. To quantify protein spots, a matchset of all gels was made and the absorbance of individual protein spots from 2-D gels was measured. Total density in the gel image was used for normalization of spots. In this method, the raw quantity of each spot in a member gel is divided by the total intensity value of all the pixels in the image. This model assumes that the total density of an image (i.e. background density plus spot intensity) will be relatively consistent from gel to gel. Student's t-test and Mann-Whitney Signed-Rank test were used for analysis of protein expression. A probability of $p<0.05$ was considered statistically significant. Differentially regulated spots were excised and transferred to 96-well microtiter plates (Thermo) by the EXQuest™ spot cutter (BioRad) for further identification by mass spectrometry.

2.8 Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry

Each of the CBB-stained gel plugs was washed twice with 15 µL ammonium hydrogencarbonate solution (100 mmol/L) and then dehydrated twice with 15 µL acetonitrile (ACN). Proteins were digested with a total of 100 ng trypsin (sequencing grade, Promega) per gel plug at 37°C for 4 h. Resulting peptides were extracted with 1% trifluoroacetic acid (TFA) in an ultrasonic bath (Sonorex, Super RK 514 BH, Bandelin) for 5 min.

1.5 µL of the peptide extracts were manually spotted onto a 600 µm/384 well AnchorChip™ sample target (Bruker Daltonics) and dried at room temperature. The MALDI target was covered with a thin layer of α-cyano-4-hydroxycinnamic acid (CHCA) matrix dissolved in 97% Acetone/ 3% TFA 0.1% to saturation. To enable MS/MS measurement matrix recristallization was performed with 0.8 µL of 60% ethanol/ 30% acetone/ 10% TFA 1%. MALDI mass spectra were recorded using an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 384-sample scout source. An external peptide calibration standard containing the following fragments was used to calibrate the instrument: angiotensin II ($[M+H]^+$ 1046.54); angiotensin I ($[M+H]^+$ 1296.68); substance P ($[M+H]^+$ 1347.74); bombesin ($[M+H]^+$ 1619.82); ACTH clip 1–17 ($[M+H]^+$ 2093.09); ACTH clip 18–39 ($[M+H]^+$ 2465.20); somatostatin 28 ($[M+H]^+$ 1347.47) (Bruker Daltonics). Mass spectra were acquired in an automatic mode using the AutoXecute™ module of the FlexControl™ software (version 2.4, Bruker Daltonics) and using the three most abundant peptide signals of the corresponding peptide mass fingerprint (PMF) and peptide fragmentation fingerprint (PFF) spectrum. Spectra were analyzed using the FlexAnalysis™ software (version 2.4, Bruker Daltonics). The Swiss-Prot database employing the MASCOT program (version 2.0, Matrix Science, in-house server) was used for the search of peptide masses to identify proteins. Database searches were performed taking into account carbamidomethyl modification of cysteines and possible oxidation of methionine. One missed cleavage was allowed. A mass inaccuracy of ≤ 100 ppm was allowed for PMF. For PFF, a mass inaccuracy of ≤ 70 ppm was allowed for peptide masses and their fragments, respectively. Identified

proteins were sent to the Proteinscape™ database (Protagen) and checked individually for further consideration.

2.9 Western blotting

Expression of selected proteins was further confirmed by Western blotting. Serum samples (50 µg) used for 2-DE were run on 12% or 15% gels by SDS-PAGE, blotted onto PVDF membranes and blocked with 10% Rotiblock™ (Roth) in TBS for 1 hour at room temperature. Primary antibodies were diluted in TBS with 1% Rotiblock for 1h each. The membranes were incubated with goat anti-amphiregulin (1:250, Santa Cruz, product no. sc-5797), rabbit anti-ApoA1 (1:200, Santa Cruz, product no. sc-30089), mouse anti-ApoM (1:200, BD Biosciences, product no. 612332), rabbit anti-A-raf (1:200, Santa Cruz, product no. 28772), rabbit anti-EGF (1:200, Calbiochem, product no. GF07L), goat anti-transthyretin (1:200, product no. sc-8104) and mouse anti-α-tubulin (1:200, product no. sc-58667). HRP-conjugated IgGs (1:10000, Chemicon) were used as secondary antibodies. Rat liver total extracts and HeLa total extracts were used as positive controls. α-tubulin was used as a loading control. Membranes were washed three times with TBS/0.1% Tween between each antibody incubation and detected with enhanced chemiluminescence (Perkin Elmer) for 60 min with a CF440 imager (Kodak).

3 Results and Discussion

3.1 Histopathology of the lung

At early stages of disease the SP-C/IgEGF transgenic mice developed hyperplasia (Fig. 1b) and dysplasia (Fig. 1c) while at advanced stages adenocarcinomas could be identified (Fig. 1d and 1e). As evidenced by histopathology, the lung displayed multiple foci which were indicated by the increased cellularity in the alveoli. The alveoli exhibit a hyperplastic and dysplastic epithelium consisting of cuboidal cells lining the alveolar septae and ducts.

3.2 Separation of serum proteins

To extract proteins from mouse serum a thiourea-containing lysis buffer was used as recently reported. Analyzing the serum proteome is challenging due to interference by high-abundant proteins such as albumin, antitrypsin, transferrin and immunoglobulins. Pre-fractionation and depletion of these proteins is useful for proteome profiling, but may also introduce bias due to depletion of low-abundant proteins [17]. Consequently, we did not pre-fractionate our protein extracts. Instead proteins were separated within the pH ranges 3-10, 4-7 and 5-8 and visualized with the CCB stain. Approximately 450 (pH 3-10) and 250 (pH 4-7 and 5-8) distinct spots per gel were detected, respectively. Fig. 2 depicts representative serum proteome maps of EGF transgenic mice.

3.3 Identification of serum proteins

Ninety-six 2-D gels were stained with the CCB method and studied by image analysis. Protein spots of representative gels were analyzed by MALDI-MS after tryptic in-gel digest. Identification of proteins was based on Swiss-Prot database entries with the MASCOT search engine. Overall, sixty eight serum proteins could be annotated (Tab. 1). In Supplementary Table 1 the MASCOT score, sequence coverage and the most informative peptide sequences identified by MS and MS/MS are listed. To the best of our knowledge, nine of the identified proteins have not been reported so far in mouse serum or plasma of healthy animals [18-20]. These proteins include the peroxisomal acyl-coenzyme A oxidase 3 (Swiss-Prot ID Q9EPL9, spot no. 9), autoimmune regulator protein (Q9Z0E3, spot no. 12), carnithine palmitoyltransferase II (P52825, spot no. 28), heparin-binding growth factor (P15655, spot no. 31), Ig gamma-3 chain C region, secreted form (P22436, spot no. 32), Ig kappa chain V-III region PC 7132 (P01655, spot no. 44), myosin-14 (Q6URW6, spot no. 51), tripartite motif-containing protein 30 (P15533, spot no. 63) and vacuolar protein sorting-associated protein 28 homologue (Q9D1C8, spot no. 65).

3.4 Regulation of serum proteins in an EGF transgenic disease model

Tab. 2a-c report regulation of proteins when serum extracts of wildtype and disease bearing proteomes were compared. Prominent examples of regulated proteins are depicted in Fig. 3. Protein expression was confirmed by Western blotting (Fig. 4). A summary of regulated proteins at different pH ranges is given in Fig. 5a and 5b and significantly regulated proteins are discussed below for their disease association.

3.4.1 Regulation of signaling molecules in lung dysplasia

As shown in Tab. 1 and Fig. 3 we identified **gelsolin** (GSN, Swiss-Prot ID P13020, spot no. 34). This protein is an actin-binding protein and a key regulator of actin (Swiss-Prot ID P63260, spot no. 10) dynamics, i.e. filament assembly and disassembly. GSN is one of the most potent members of the gelsolin/villin superfamily. GSN has been located in cytosol and mitochondria but in plasma as well. Along with Arp3, cortactin, and Rho GTPases GSN plays a role in podosome formation [21]. Prior to cell death, mitochondria lose membrane potential and become more permeable. Normally, GSN inhibits the release of cytochrome C, blocking the signal amplification that would have led to apoptosis. Thus, GSN inhibits apoptosis by stabilizing the mitochondria [22]. An impairment of GSN has been shown to cause increased permeability of the vascular pulmonary barrier in mice, suggesting that GSN is important in the response to lung injury [23, 24]. The prognostic significance of GSN in NSCLC was shown by Yang and co-workers [25]. In our study GSN was significantly ($p<0.05$) upregulated, albeit by approximately 2-fold in mice with dysplasia.

We also identified **fetuin B** (Swiss-Prot ID Q9QXC1, spot no. 30) as regulated. Notably, fetuins, which are part of the cystatin superfamily, have been implicated in several functions, i.e. osteogenesis and bone resorption, regulation of the insulin and hepatocyte growth factor receptors. In an effort to identify hepatic genes preferentially expressed during acute inflammation, Olivier and co-workers

identified rat hepatic fetuin B to be repressed [26]. Furthermore, the 382-amino acid human fetuin B protein shares >60% sequence and structural similarity with fetuin A. Overexpression of fetuin B in skin squamous carcinoma cells led to suppression of tumour growth in nude mice [27]. Here we report fetuin B to be marginally, but statistically significantly ($p<0.05$) upregulated in mice with dysplasia of the lung.

Another signaling protein with marginal upregulation at early stages of disease, but with >3-fold overexpression in tumour bearing mice ($p<0.05$) was **alpha-2 macroglobulin** (Swiss-Prot ID Q61838, A2M, spot no. 8-A and 8-B). This protein is a known proteinase inhibitor and positive acute phase reactant which acts as an inhibitor of coagulation by inhibiting thrombin and as an inhibitor of fibrinolysis by inhibiting Plasmin. A2M was overexpressed in the serum of c-raf and c-myc transgenic mice that developed atypical adenomatous hyperplasias (AAH) and bronchiolo-alveolar carcinomas (BAC), respectively. We thus propose this protein as a candidate biomarker for the early detection and monitoring of lung cancer [3, 28].

As reported in Tab. 2a we identified **a-raf** (ARAF, Swiss-Prot ID P04627, spot no. 21) as regulated. This protein is a member of the RAF serine/threonine kinase family. Specifically, the RAF family of genes encode cytoplasmic protein serine/threonine kinases that play a critical role in cell growth and development [29]. RAF kinases are key molecules for the mitogenic signal transduction pathway and connect receptor tyrosine kinases through MEK and ERK with nuclear transcription factors. There is evidence for the serine/threonine kinases of the RAF family to be regulated in lung cancer when overexpressed or mutated [30]. However, here we show the ARAF kinase to be downregulated by about 2-fold ($p<0.05$) in transgenic mice with lung dysplasia by 2-DE (Fig. 3) and Western blotting (Fig. 4). Although in the mouse ARAF is predominantly expressed in urogenital tissues, it was suggested that in the absence of ARAF, the caveolar coat is less stable or inefficiently assembled [31]. These observations revealed new insights into caveolae trafficking and suggested that the dynamic properties of caveolae and their transport competence are regulated by different kinases

operating at several levels.

We also observed regulation of **plasminogen** (PLG, Swiss-Prot ID P20918, spot no. 54), a circulating zymogen that is converted to the active enzyme plasmin by cleavage of the peptide bond between the amino acids Arg560 and Val561. This reaction is mediated by the urokinase PLG activator (PLAU) and the tissue PLG activator (TPA, PLAT). Plasmin, like trypsin, belongs to the family of serine proteinases and mainly dissolves fibrin clots. The PLG activation system in lung cancer was described elsewhere [32] and substantial anti-proliferative and pro-apoptotic effects of plasminogen in tumour cells were recently reported for a lung cancer model [33]. Note, PLG was significantly ($p<0.05$) downregulated by nearly 2-fold in transgenic mice at early stages of tumourigenesis, but not regulated at late stages of disease.

3.4.2 Regulation of transporters in lung dysplasia

The **vitamin D binding protein** (VTDB, Swiss-Prot ID P21614, spot no. 66) was statistically significantly ($p<0.05$) upregulated in disease bearing mice with dysplasia of the respiratory epithelium. This multifunctional protein, also known as Gc globulin, is found in plasma, ascitic fluid, cerebrospinal fluid, urine and on the surface of many cell types. In plasma, VTDB carries the vitamin D sterols and prevents polymerization of actin (Swiss-Prot ID P63260, spot no. 10) by binding to its monomers. Overexpression of VTDB in breast cancer was reported elsewhere [34, 35]. Its regulation in lung cancer was described previously [3].

Additionally, we observed the **vacuolar protein sorting-associated protein 28 homologue** (Vps28, spot no. 65) to be significantly upregulated by >5-fold ($p<0.05$) at early stages of cancer (12-14 months). Notably, the endosomal sorting complex required for transport (ESCRT) complexes are central to receptor downregulation and lysosome biogenesis. For instance, the yeast ESCRT-I complex contains the Vps23, Vps28, and Vps37 proteins, and its assembly is directed by the C-terminal steadiness box of Vps23, the N-terminal half of Vps28 and the C-terminal half of Vps37 [36]. Previously, Olabisi and colleagues reported expression

of the aberrant protein Bcr in patients with chronic myelogenous leukemia (CML). Bcr was shown to play an important role in cellular trafficking of the EGFR while Vps28 was found to interact with Bcr [37]. So far, little is known about Vps28 and its regulation in lung cancer. Here we propose Vps28 as a diagnostic marker for lung dysplasia.

Furthermore, we identified the negative acute phase protein **transthyretin** (TTHY, Swiss-Prot ID P07309, spot no. 64) to be highly significantly repressed by >19-fold ($p<0.05$) in disease bearing mice with lung dysplasia. This finding was further validated by Western blotting (Fig. 4). Specifically, TTHY carries thyroid hormones from blood to tissues and interacts with the plasma retinol binding protein (RETBP, Swiss-Prot ID Q00724, spot no. 56) to enable transportation of retinol [38]. Lower levels of RETBP are observed if TTHY expression is decreased as demonstrated in a recent serum proteomic study of liver and lung cancer [39]. Likewise, expression of RETBP was marginally, but not significantly reduced in mice with dysplasia (data not shown). Being a negative acute phase reactant, but induced in tumour bearing mice makes RETBP a likely candidate biomarker of disease and has been shown to be regulated in c-myc transgenic lung tumour mice as well. In the past we observed regulation of TTHY in lung cancer upon targeted activation of c-myc and c-raf [3, 28]. In clinical studies TTHY was also proposed as a serum biomarker for the diagnosis of lung cancer [40-42].

3.4.3 Regulation of apolipoproteins in lung dysplasia

The pulmonary surfactant is a surface-active lipoprotein complex formed by alveolar type II cells (AT-II). The surfactant comprising proteins and lipids have both a hydrophilic and a hydrophobic region. By adsorption to the air-water interface of alveoli with the hydrophilic headgroups in the water and the hydrophobic tails facing towards the air, the main lipid component of surfactant, dipalmitoylphosphatidylcholine (DPPC), reduces surface tension [43]. Lung surfactant contains >90% lipids, such as DPPC, whereas the remaining 10% of surfactant are proteins, such as the surfactant protein C (SP-C). An increase of SP-C levels in the lung of tumour bearing mice has been reported elsewhere [44].

Notably, apolipoproteins are proteins that bind to lipids and form particles, which transport lipids from the intestine to the liver and from the liver to tissues, including adipocytes, heart, muscle, breast and lung tissues. Apolipoproteins also serve as enzyme co-factors, lipid transfer carriers and receptor ligands that regulate the metabolism of lipoproteins and their uptake in tissues. For instance, the low density lipoprotein receptor-related protein 1 (LRP1), i.e. a cellular membrane receptor of the low density lipoprotein (LDL) receptor family, is a multiligand receptor with important signaling function [45]. Apolipoprotein E (Swiss-Prot ID P08226, spot no. 18) interacts with LRP1 to mediate uptake of chylomicron remnants into the liver. There is evidence for regulation of apolipoproteins in a large number of malignancies, such as lung cancer.

In particular, we observed expression of the apolipoprotein A-I (ApoA1, Swiss-Prot ID Q00623, spot no. 15), apolipoprotein A-IV (ApoA4, Swiss-Prot ID P06728, spot no. 16), apolipoprotein H (ApoH, Swiss-Prot ID Q01339, spot no. 19) and apolipoprotein M (ApoM, Swiss-Prot ID Q9Z1R3, spot no. 20) to be significantly regulated in disease bearing mice.

Specifically, **ApoA1** belongs to the ApoA1/A4/E protein family and is primarily produced in the liver and the intestine. ApoA1 can be found in the extracellular space and takes part in cholesterol absorption. Particularly, the ATP-binding cassette transporter A1 (ABCA1) has been found to efflux cholesterol indirectly to ApoA1 in plasma membranes [46]. By quantitative proteomic approaches regulation of ApoA1 in association with lung cancer was reported [44, 47] and induction of ApoA1 supports its use as a serum marker for the early detection of brain metastases in lung cancer patients [48]. Here we report ApoA1 expression to be significantly induced by nearly 6-fold at early stages, but reduced by >3-fold ($p<0.05$) at late stages of lung cancer.

ApoA4 may play a role in secretion and catabolism of very low density lipoproteins (VLDL). ApoA4 is required for efficient activation of lipoprotein lipase by apolipoprotein C-II and is a potent activator of the lecithin-cholesterin acyltransferase (LCAT) [49]. Furthermore, ApoA4 is a major component of high

density lipoprotein (HDL) and chylomicron and was found to be upregulated in pancreatic cancer [50]. Based on two-dimensional fluorescence difference gel-electrophoresis (2-D DIGE) ApoA4 expression was found to be increased in serum of patients with squamous cell carcinoma of the lung [51]. ApoA4 was, however, downregulated by nearly 2-fold and >1.2-fold ($p<0.05$) in EGF transgenic mice with dysplasia and lung tumours, respectively.

ApoH, also known as beta-2 glycoprotein-1 was repressed in transgenic mice at early stages of disease. This protein binds to various kinds of negatively charged substances such as heparin, phospholipids and dextran sulfate. Through binding to phospholipids on the surface of damaged cells, ApoH may inhibit activation of the intrinsic blood coagulation cascade [52]. ApoH is synthesized in the liver and secreted into plasma. As reported by others, ApoH induction may be involved in blocking angiogenic processes in bladder cancer [53, 54]. In our recent study on c-myc lung tumour bearing mice we proposed ApoH as a candidate biomarker [28]. ApoH regulation in serum of transgenic mice at late stages of disease remained unchanged when compared to control mice.

A further protein of considerable interest is **ApoM**, i.e. a 26-kDa protein expressed mainly in the liver and kidneys [55]. It is predominantly found as a part of HDL particle and belongs to the lipocalin superfamily. Substantially decreased ApoM levels in ApoA1-deficient mice suggest a connection between ApoM and ApoA1 metabolism [56]. Recently, upregulation of ApoM was reported in serum of patients with hepatocellular carcinoma (HCC) [57] and in serum of transgenic mice with HCC [8]. To the best of our knowledge, regulation of ApoM is a novel finding in terms of lung neoplasias and we found this lipoprotein to be significantly ($p<0.05$) downregulated by >6-fold and >2-fold in serum of disease bearing mice at early and late stages of disease, respectively.

3.4.4 Serum markers of aberrant EGF activity

Recently, our laboratory reported mapping of the serum proteome of hepatocellular carcinoma (HCC) induced by targeted overexpression of EGF to liver cells of transgenic mice [8]. We now compared the results from the present study with previous findings in an EGF liver cancer model and identified four proteins in common, i.e. A2M, ApoA1, ApoM and GPX3. Notably, in both transgenic disease models, serum EGF levels were increased. It is of considerable importance that Xu and colleagues reported repressed ApoM mRNA levels in HepG2 cells upon treatment with TGF-α, HNF and EGF, respectively [58]. In another clinical study lower levels of ApoA1 were observed upon high serum levels of EGF [59]. Thus, these proteins may serve as candidate markers of disease linked to aberrant EGFR activity.

3.4.5 Expression of disease regulated proteins in three different transgenic disease models of lung cancer

Previously, we reported expression of regulated proteins in the serum of different transgenic mouse models of lung cancer. Comparison of regulated proteins from EGF transgenic mice with those of c-raf and c-myc [3, 28] confirmed regulation of A2M and TTHY to be in common. Additionally, ApoA1, ApoH and **glutathione peroxidase 3** (GPX3, spot no. 35) were commonly regulated in c-myc and EGF transgenic mice, while α-1 antitrypsin and properdin were in common for c-raf and c-myc tumour bearing mice. In particular, GPX3 is a protector of cells and enzymes from oxidative damage, by catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxide, by glutathione. We previously reported increased expression of GPX3 in c-myc lung tumour bearing mice, while upregulation of GPX3 was observed in ovarian cancer and NSCLC [60, 61]. These findings make this protein a likely candidate biomarker of lung cancer.

Finally, VTDB was similarly in c-raf and EGF disease bearing mice (Fig. 6). These findings are encouraging and evidence broad use of the proposed serum

biomarkers in disease models of aberrant c-raf, c-myc and EGFR tyrosine kinase (Swiss-Prot ID Q01279, spot no. 29) activity all of which play essential roles in lung cancer [62, 63]. Furthermore, amphiregulin and epiregulin activate EGFR and are often regulated in lung tumours [64, 65]. Exclusive expression of amphiregulin and EGF was confirmed in diseased animals as evidenced by Western immunoblotting (Fig. 4), whereas epiregulin was not detected in serum. Notably, an anti-apoptotic effect of amphiregulin in lung cancer and liver injury has been reported [66, 67]. Serum levels of soluble EGFR and EGF as markers for NSCLC were also proposed by Lemos-González *et al.* [68].

4 Concluding remarks

Based on our previous studies on the SP-C/IgEGF transgenic mouse model [13, 14] we report disease regulated serum proteins, that function as transporters, signaling molecules and apolipoproteins and are associated with distinct neoplastic changes of the respiratory epithelium at early and later stages of lung cancer. As shown by histopathology transgenic mice developed dysplastic changes of the respiratory epithelium followed by lung tumors at late stages. We compared the serum proteome map established herein with those previously published [3, 28]. Some of the identified regulated proteins are known as acute phase reactants (APP), of which α-2 macroglobulin (positive APP) and transthyretin (negative APP) are prominent examples and fit best with studies by others and our own findings [8, 39]. Regulation of the apolipoproteins A1, A4, H and M is an important finding with apolipoprotein A1 and H being reported as upregulated in human lung cancer [47, 48, 53]. We suggest ApoM as a diagnostic marker for lung neoplasias at early and late stages of disease. Its regulation correlates well with other human malignancies [57]. While gelsolin, plasminogen and vitamin D binding protein are known to be regulated in bronchiolo-alveolar malignancies [25, 33], regulation of fetuin B was shown to be differentially expressed in skin squamous carcinoma [27]. Downregulation of a-raf and upregulation of Vps28 are novel in terms of early stages of lung cancer, e.g. atypical adenomatous hyperplasia. Recently, Gesner *et al.* observed increased expression of glutathione peroxidase in NSCLC patients [61]. Regulation of GPX3 in our present study correlates well with our previous findings in c-myc transgenic mice and makes this protein a likely candidate biomarker of disease. Furthermore, we view EGF and amphiregulin as particularly relevant for the detection of early stages of disease. A comparison of EGF disease bearing mice with c-raf and c-myc transgenic animals, all of which developed different types of lung cancer, identified common regulation of α-2 macroglobulin and transthyretin. Taken collectively, our EGF disease model enabled identification of serum biomarker candidates of dysplasia in response to aberrant EGFR activity [69]. The identified proteins warrant in depth clinical evaluation for the early detection of human lung malignancies.

Figure captions

Figure 1:

(a) Histology of lung with thin alveolar septae in healthy non-transgenic mice. (b) Lung of a SP-C/IgEGF transgenic mouse with multifocal hyperplasia of the respiratory epithelium, indicated by an increased alveolar cellularity. (c) Alveolar dysplasia in lung of transgenic mice. (d, e) Adenocarcinoma of transgenic mice.

Figure 2:

Representative 2-DE maps displaying the serum proteome of SP-C/IgEGF transgenic mice with pH ranges from 3-10, 4-7 and 5-8.

Figure 3:

Examples of regulated serum proteins in SP-C/IgEGF transgenic and wildtype aged matched control mice. Representative gels from each group are shown. Protein spots of interest are marked by circles.

Figure 4:

Western immunoblotting of amphiregulin, apolipoprotein A-I, apolipoprotein M, a-raf and transthyretin in serum of EGF transgenic mice compared to wildtype control mice. EGF and amphiregulin were exclusively expressed in transgenic animals. α-tubulin was used as a loading control. N.D.: positive control was not detected, but no other protein band except that of transthyretin (55 kDa) was detected. A part of the image with protein bands of interest cropped and marked by molecular weights is shown.

Figure 5a:

Fold-changes of disease regulated proteins in serum of EGF transgenic mice, aged 12-14 months, with dysplasia of the lung compared to control samples without neoplasias of the lung. Three pH ranges, i.e. 3-10, 4-7 and 5-8 were applied to 2-DE. Values are mean ± SD. *$p<0.05$. A: signaling molecules, B: transporters, C: apolipoproteins.

Figure 5b:

Fold-changes of disease regulated proteins in serum of EGF transgenic mice with lung tumours, aged 18-20 months, compared to control samples without neoplasias of the lung. Values are mean ± SD. *$p<0.05$.

Figure 6:

Comparison of regulated serum proteins from three transgenic mouse models with targeted overexpression of c-raf, c-myc and EGF, respectively, to bronchiolo-alveolar epithelium by use of the surfactant protein-C (SP-C) promoter. All models developed malignant transformation of alveolar type II cells. **A** (c-raf transgenic mouse model): AAT 1-3, EGFR, Ig γ-2B chain C region, α-globin, Ig J chain, Ig κ chain C region, Ig κ chain V-III region MOPC 70, MUP2, MUP8, sodium- and chloride-dependent GABA transporter 3; **B** (c-myc transgenic mouse model): orosomucoid 8, AAT 1-1, ApoC3, ApoE, RETBP, SAP; **C** (EGF transgenic mouse model): ApoA4, ApoM, a-raf, fetuin B, GSN, PLG, VPS28; **AB**: AAT 1-6, properdin; **AC**: VTDB, **BC**: ApoA1, ApoH, GPX3; **ABC**: A2M, TTHY.

Table captions

Table 1:

Protein identification in 2-DE maps of mouse serum proteins in SP-C/IgEGF transgenic mice. A total of sixty eight proteins were identified by MALDI-MS. See Supplementary Table 1 for detailed information.

Table 2a and 2b:

Expression profiles of significantly regulated proteins from 2-D gels at pH 3-10, 4-7 and 5-8. Quantification of protein abundance was analyzed using the PDQuest 2-D software (BioRad) by measurement of the normalized OD (arbitrary units) of each protein spot. The change in abundance of the proteins is expressed by the calculated ratio (T/C) between mean values of spot volumes from transgenic (T) over that from healthy control (C) samples. Student's t-test and Mann-Whitney Signed-Rank tests were used for calculation of p-values, while $p<0.05$ was considered statistically significant. %RSD: percentual relative standard deviation. The error is calculated based on the error propagation equation:

$$\Delta z = \sqrt{\left(\frac{\partial z}{\partial y}\right)^2 \Delta y^2 + \left(\frac{\partial z}{\partial x}\right)^2 \Delta x^2}$$

Table 2c:

Overview of representative protein regulations (fold-changes) in EGF transgenic mice with lung dysplasia and lung cancer. ↑: up-regulation, ↓: down-regulation, ↔: no regulation, N.D.: spot not detected/not quantifiable

Supplementary table 1:

Serum proteins of SP-C/IgEGF transgenic mice. The MASCOT score (PMF), ions score (PFF), the number of identified peptides, their sequence, the sequence coverage of the best hits and supporting information are shown for each identified protein. *M*r and p*I* are based on the theoretical values of the precursor. The value from one typical spot was given if many spots were assigned as one number (protein). O@M: oxidation at the amino acid methionine.

Acknowledgements

The authors are grateful to Dr. Roman Halter for providing transgenic mice. Dr. Dörte Solle and Stefanie Marschke is thanked for their valuable comments and technical advice.

The authors have declared no conflict of interest.

5 References

[1] Hirsch FR, Scagliotti GV, Langer CJ, Varella-Garcia M, Franklin WA. Epidermal growth factor family of receptors in preneoplasia and lung cancer: perspectives for targeted therapies. *Lung Cancer* **2003**, *41 Suppl 1*, S29-S42. Review.

[2] Tiseo M, Loprevite M, Ardizzoni A. Epidermal growth factor receptor inhibitors: a new prospective in the treatment of lung cancer. *Curr. Med. Chem. Anticancer Agents* **2004**, *4*, 139-148. Review.

[3] Chatterji B, Borlak J. Serum proteomics of lung adenocarcinomas induced by targeted overexpression of c-raf in alveolar epithelium identifies candidate biomarkers. *Proteomics* **2007**, *7*, 3980-3991.

[4] Duan X, Yarmush DM, Jayaraman A, Yarmush ML. Dispensable role for interferon-gamma in the burn-induced acute phase response: a proteomic analysis. *Proteomics* **2004**, *4*, 1830-1839.

[5] Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. *Nat. Rev. Cancer* **2007**, *7*, 169-181. Review.

[6] Tönjes RR, Löhler J, O'Sullivan JF, Kay GF, Schmidt GH, Dalemans W, Pavirani A, Paul D. Autocrine mitogen IgEGF cooperates with c-myc or with the Hcs locus during hepatocarcinogenesis in transgenic mice. *Oncogene* **1995**, *10*, 765-768.

[7] Borlak J, Meier T, Halter R, Spanel R, Spanel-Borowski K. Epidermal growth factor-induced hepatocellular carcinoma: gene expression profiles in precursor lesions, early stage and solitary tumours. *Oncogene* **2005**, *24*, 1809-1819.

[8] Gazzana G, Borlak J. Mapping of the serum proteome of hepatocellular carcinoma induced by targeted overexpression of epidermal growth factor to liver cells of transgenic mice. *J. Proteome Res.* **2008**, *7*, 928-937.

[9] Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **2007**, *26*, 3291-3310. Review.

[10] Gilmore JL, Scott JA, Bouizar Z, Robling A, Pitfield SE, Riese DJ 2nd, Foley J. Amphiregulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells. *Breast Cancer Res. Treat.* **2008**, *110*, 493-505.

[11] Hijiya N, Miyawaki M, Kawahara K, Akamine S, Tsuji K, Kadota J, Akizuki S, Uchida T, Matsuura K, Tsukamoto Y, Moriyama M. Phosphorylation status of epidermal growth factor receptor is closely associated with responsiveness to gefitinib in pulmonary adenocarcinoma. *Hum. Pathol.* **2008**, *39*, 316-323.

[12] Schuchardt S, Borlak J. Quantitative mass spectrometry to investigate epidermal growth factor receptor phosphorylation dynamics. *Mass Spectrom. Rev.* **2008**, *27*, 51-65.

[13] Ehrhardt A, Bartels T, Geick A, Klocke R, Paul D, Halter R. Development of pulmonary bronchiolo-alveolar adenocarcinomas in transgenic mice overexpressing murine c-myc and epidermal growth factor in alveolar type II pneumocytes. *Br J Cancer* **2001**, *84*, 813-818.

[14] Ehrhardt A, Bartels T, Klocke R, Paul D, Halter R. Increased susceptibility to the tobacco carcinogen 4-[methylnitrosamino]-1-[3-pyridyl]-1-butanone in transgenic mice overexpressing c-myc and epidermal growth factor in alveolar type II cells. *J. Cancer Res. Clin. Oncol.* **2003**, *129*, 71-75.

[15] Lantuéjoul S, Salameire D, Salon C, Brambilla E. Pulmonary preneoplasia - sequential molecular carcinogenetic events. *Histopathology* **2009**, *54*, 43-54.

[16] Brambilla C, Fievet F, Jeanmart M, de Fraipont F, Lantuejoul S, Frappat V, Ferretti G, Brichon PY, Moro-Sibilot D. Early detection of lung cancer: role of biomarkers. *Eur. Respir. J. Suppl.* **2003**, *39*, 36s-44s. Review.

[17] Ritorto MS, Borlak J. A simple and reliable protocol for mouse serum proteome profiling studies by use of two-dimensional electrophoresis and MALDI TOF/TOF mass spectrometry. *Proteome Sci.* **2008**, *6*, 25.

[18] Lai KK, Kolippakkam D, Beretta L. Comprehensive and quantitative proteome profiling of the mouse liver and plasma. *Hepatology* **2008**, *47*, 1043-1051.

[19] Hood BL, Zhou M, Chan KC, Lucas DA, Kim GJ, Issaq HJ, Veenstra TD,

Conrads TP. Investigation of the mouse serum proteome. *J. Proteome Res.* **2005**, *4*, 1561-1568.

[20] Anderson NL, Polanski M, Pieper R, Gatlin T, Tirumalai RS, Conrads TP, Veenstra TD, Adkins JN, Pounds JG, Fagan R, Lobley A. The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol. Cell. Proteomics* **2004**, *3*, 311-326.

[21] Chellaiah MA. Regulation of podosomes by integrin alphavbeta3 and Rho GTPase-facilitated phosphoinositide signaling. *Eur. J. Cell Biol.* **2006**, *85*, 311-317. Review.

[22] Koya RC, Fujita H, Shimizu S, Ohtsu M, Takimoto M, Tsujimoto Y, Kuzumaki N. Gelsolin inhibits apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release. *J. Biol. Chem.* **2000**, *275*, 15343-15349.

[23] Lind SE, Smith DB, Janmey PA, Stossel TP. Depression of gelsolin levels and detection of gelsolin-actin complexes in plasma of patients with acute lung injury. *Am. Rev. Respir. Dis.* **1988**, *138*, 429-434.

[24] Becker PM, Kazi AA, Wadgaonkar R, Pearse DB, Kwiatkowski D, Garcia JG. Pulmonary vascular permeability and ischemic injury in gelsolin-deficient mice. *Am. J. Respir. Cell. Mol. Biol.* **2003**, *28*, 478-484.

[25] Yang J, Ramnath N, Moysich KB, Asch HL, Swede H, Alrawi SJ, Huberman J, Geradts J, Brooks JS, Tan D. Prognostic significance of MCM2, Ki-67 and gelsolin in non-small cell lung cancer. *BMC Cancer* **2006**, *6*, 203.

[26] Olivier E, Soury E, Ruminy P, Husson A, Parmentier F, Daveau M, Salier JP. Fetuin-B, a second member of the fetuin family in mammals. *Biochem. J.* **2000**, *350 Pt 2*, 589-597.

[27] Hsu SJ, Nagase H, Balmain A. Identification of Fetuin-B as a member of a cystatin-like gene family on mouse chromosome 16 with tumor suppressor activity. *Genome* **2004**, *47*, 931-946.

[28] Chatterji B, Borlak J. A 2-DE MALDI-TOF study to identify disease regulated serum proteins in lung cancer of c-myc transgenic mice. *Proteomics* **2009**, *9*, 1044-1056.

[29] Magnuson NS, Beck T, Vahidi H, Hahn H, Smola U, Rapp UR. The Raf-1

serine/threonine protein kinase. *Semin. Cancer Biol.* **1994**, *5*, 247-253. Review.

[30] Gollob JA, Wilhelm S, Carter C, Kelley SL. Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduction pathway. *Semin. Oncol.* **2006**, *33*, 392-406. Review.

[31] Pelkmans L, Zerial M. Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. *Nature* **2005**, *436*, 128-133.

[32] Pappot H. The plasminogen activation system in lung cancer - with special reference to the prognostic role in "non-small cell lung cancer". *APMIS Suppl.* **1999**, *92*, 1-29.

[33] Schmitz V, Raskopf E, Gonzalez-Carmona MA, Vogt A, Kornek M, Sauerbruch T, Caselmann WH. Plasminogen derivatives encoding kringles 1-4 and kringles 1-5 exert indirect antiangiogenic and direct antitumoral effects in experimental lung cancer. *Cancer Invest.* **2008**, *26*, 464-470.

[34] Pawlik TM, Hawke DH, Liu Y, Krishnamurthy S, Fritsche H, Hunt KK, Kuerer HM. Proteomic analysis of nipple aspirate fluid from women with early-stage breast cancer using isotope-coded affinity tags and tandem mass spectrometry reveals differential expression of vitamin D binding protein. *BMC Cancer* **2006**, *6*, 68.

[35] Abbas S, Linseisen J, Slanger T, Kropp S, Mutschelknauss EJ, Flesch-Janys D, Chang-Claude J. The Gc2 allele of the vitamin D binding protein is associated with a decreased postmenopausal breast cancer risk, independent of the vitamin D status. *Cancer Epidemiol. Biomarkers Prev.* **2008**, *17*, 1339-1343.

[36] Kostelansky MS, Sun J, Lee S, Kim J, Ghirlando R, Hierro A, Emr SD, Hurley JH. Structural and functional organization of the ESCRT-I trafficking complex. *Cell* **2006**, *125*, 113-126.

[37] Olabisi OO, Mahon GM, Kostenko EV, Liu Z, Ozer HL, Whitehead IP. Bcr interacts with components of the endosomal sorting complex required for transport-I and is required for epidermal growth factor receptor turnover. *Cancer Res.* **2006**, *66*, 6250-6257.

[38] Yamamoto Y, Yoshizawa T, Kamio S, Aoki O, Kawamata Y, Masushige S,

Kato S. Interactions of transthyretin [TTR] and retinol-binding protein [RBP] in the uptake of retinol by primary rat hepatocytes. *Exp. Cell. Res.* **1997**, *234*, 373-378.

[39] Li SQ, Yun J, Xue FB, Bai CQ, Yang SG, Que HP, Zhao X, Wu Z, Wang Y, Liu SJ. Comparative proteome analysis of serum from acute pulmonary embolism rat model for biomarker discovery. *J. Proteome Res.* **2007**, *6*, 150-159.

[40] Suresh UR, Wilkes S, Hasleton PS. Prealbumin in the diagnosis of bronchopulmonary carcinoid tumours. *J. Clin. Pathol.* **1991**, *44*, 573-575.

[41] Papla B, Rudnicka L. Primary amyloid tumors of the lungs--six cases. *Pol. J. Pathol.* **2005**, *56*, 197-202.

[42] Liu L, Liu J, Dai S, Wang X, Wu S, Wang J, Huang L, Xiao X, He D. Reduced transthyretin expression in sera of lung cancer. *Cancer Sci.* **2007**, *98*, 1617-1624.

[43] Possmayer F, Nag K, Rodriguez K, Qanbar R, Schürch S. Surface activity in vitro: role of surfactant proteins. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **2001**, *129*, 209-220. Review.

[44] Kassie F, Anderson LB, Scherber R, Yu N, Lahti D, Upadhyaya P, Hecht SS. Indole-3-carbinol inhibits 4-[methylnitrosamino]-1-[3-pyridyl]-1-butanone plus benzo[a]pyrene-induced lung tumorigenesis in A/J mice and modulates carcinogen-induced alterations in protein levels. *Cancer Res.* **2007**, *67*, 6502-6511.

[45] Strickland DK, Gonias SL, Argraves WS. Diverse roles for the LDL receptor family. *Trends Endocrinol. Metab.* **2002**, *13*, 66-74. Review.

[46] Boadu E, Bilbey NJ, Francis GA. Cellular cholesterol substrate pools for adenosine-triphosphate cassette transporter A1-dependent high-density lipoprotein formation. *Curr. Opin. Lipidol.* **2008**, *19*, 270-276. Review.

[47] Maciel CM, Junqueira M, Paschoal ME, Kawamura MT, Duarte RL, Carvalho Mda G, Domont GB. Differential proteomic serum pattern of low molecular weight proteins expressed by adenocarcinoma lung cancer patients. *J. Exp. Ther. Oncol.* **2005**, *5*, 31-38.

[48] Marchi N, Mazzone P, Fazio V, Mekhail T, Masaryk T, Janigro D.

ProApolipoprotein A1: a serum marker of brain metastases in lung cancer patients. *Cancer* **2008**, *112*, 1313-1324.

[49] Takeuchi N, Matsumoto A, Katayama Y, Arao M, Koga M, Nakao H, Uchida K. Changes with aging in serum lipoproteins and apolipoprotein C subclasses. *Arch. Gerontol. Geriatr.* **1983**, *2*, 41-48.

[50] Zervos EE, Tanner SM, Osborne DA, Bloomston M, Rosemurgy AS, Ellison EC, Melvin WS, de la Chapelle A. Differential gene expression in patients genetically predisposed to pancreatic cancer. *J. Surg. Res.* **2006**, *135*, 317-322.

[51] Dowling P, O'Driscoll L, Meleady P, Henry M, Roy S, Ballot J, Moriarty M, Crown J, Clynes M. 2-D difference gel electrophoresis of the lung squamous cell carcinoma versus normal sera demonstrates consistent alterations in the levels of ten specific proteins. *Electrophoresis* **2007**, *28*, 4302-4310.

[52] Xia J, Yang QD, Yang QM, Xu HW, Liu YH, Zhang L, Zhou YH, Wu ZG, Cao GF. Apolipoprotein H gene polymorphisms and risk of primary cerebral hemorrhage in a Chinese population. *Cerebrovasc. Dis.* **2004**, *17*, 197-203.

[53] Beecken WD, Engl T, Ringel EM, Camphausen K, Michaelis M, Jonas D, Folkman J, Shing Y, Blaheta RA. An endogenous inhibitor of angiogenesis derived from a transitional cell carcinoma: clipped beta2-glycoprotein-I. *Ann. Surg. Oncol.* **2006**, *13*, 1241-1251.

[54] Sakai T, Balasubramanian K, Maiti S, Halder JB, Schroit AJ. Plasmin-cleaved beta-2-glycoprotein 1 is an inhibitor of angiogenesis. *Am. J. Pathol.* **2007**, *171*, 1659-1669.

[55] Xu N, Dahlbäck B. A novel human apolipoprotein [apoM]. *J. Biol. Chem.* **1999**, *274*, 31286-31290.

[56] Faber K, Axler O, Dahlbäck B, Nielsen LB. Characterization of apoM in normal and genetically modified mice. *J. Lipid Res.* **2004**, *45*, 1272-1278.

[57] Jiang J, Zhang X, Wu C, Qin X, Luo G, Deng H, Lu M, Xu B, Li M, Ji M, Xu N. Increased plasma apoM levels in the patients suffered from hepatocellular carcinoma and other chronic liver diseases. *Lipids Health Dis.* **2008**, *7*, 25.

[58] Xu N, Hurtig M, Zhang XY, Ye Q, Nilsson-Ehle P. Transforming growth factor-beta down-regulates apolipoprotein M in HepG2 cells. *Biochim. Biophys. Acta* **2004**, *1683*, 33-37.

[59] Lundstam U, Hägg U, Sverrisdottir YB, Svensson LE, Gan LM. Epidermal growth factor levels are related to diastolic blood pressure and carotid artery stiffness. *Scand. Cardiovasc. J.* **2007**, *41*, 308-312.

[60] Hough CD, Cho KR, Zonderman AB, Schwartz DR, Morin PJ. Coordinately up-regulated genes in ovarian cancer. *Cancer Res* **2001**, *61*, 3869-3876.

[61] Gresner P, Gromadzinska J, Jablonska E, Kaczmarski J, Wasowicz W. Expression of selenoprotein-coding genes SEPP1, SEP15 and hGPX1 in non-small cell lung cancer. *Lung Cancer* **2008**, Dec 5.

[62] Adjei AA. The role of mitogen-activated ERK-kinase inhibitors in lung cancer therapy. *Clin. Lung Cancer* **2005**, *7*, 221-223. Review.

[63] Charpidou A, Blatza D, Anagnostou E, Syrigos KN. EGFR mutations in non-small cell lung cancer--clinical implications. *In Vivo* **2008**, *22*, 529-536. Review.

[64] Ishikawa N, Daigo Y, Takano A, Taniwaki M, Kato T, Hayama S, Murakami H, Takeshima Y, Inai K, Nishimura H, Tsuchiya E, Kohno N, Nakamura Y. Increases of amphiregulin and transforming growth factor-alpha in serum as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancers. *Cancer Res.* **2005**, *65*, 9176-9184.

[65] Masago K, Fujita S, Hatachi Y, Fukuhara A, Sakuma K, Ichikawa M, Kim YH, Mio T, Mishima M. Clinical significance of pretreatment serum amphiregulin and transforming growth factor-alpha, and an epidermal growth factor receptor somatic mutation in patients with advanced non-squamous, non-small cell lung cancer. *Cancer Sci.* **2008**, Sep 22.

[66] Hurbin A, Dubrez L, Coll JL, Favrot MC. Inhibition of apoptosis by amphiregulin via an insulin-like growth factor-1 receptor-dependent pathway in non-small cell lung cancer cell lines. *J. Biol. Chem.* **2002**, *277*, 49127-49133.

[67] Berasain C, García-Trevijano ER, Castillo J, Erroba E, Santamaría M, Lee DC, Prieto J, Avila MA. Novel role for amphiregulin in protection from liver

injury. *J. Biol. Chem.* **2005**, *280*, 19012-19020.

[68] Lemos-González Y, Rodríguez-Berrocal FJ, Cordero OJ, Gómez C, Páez de la Cadena M. Alteration of the serum levels of the epidermal growth factor receptor and its ligands in patients with non-small cell lung cancer and head and neck carcinoma. *Br. J. Cancer* **2007**, *96*, 1569-1578.

[69] Thomas SM, Grandis JR, Wentzel AL, Gooding WE, Lui VW, Siegfried JM. Gastrin-releasing peptide receptor mediates activation of the epidermal growth factor receptor in lung cancer cells. *Neoplasia* **2005**, *7*, 426-431.

Figure 1

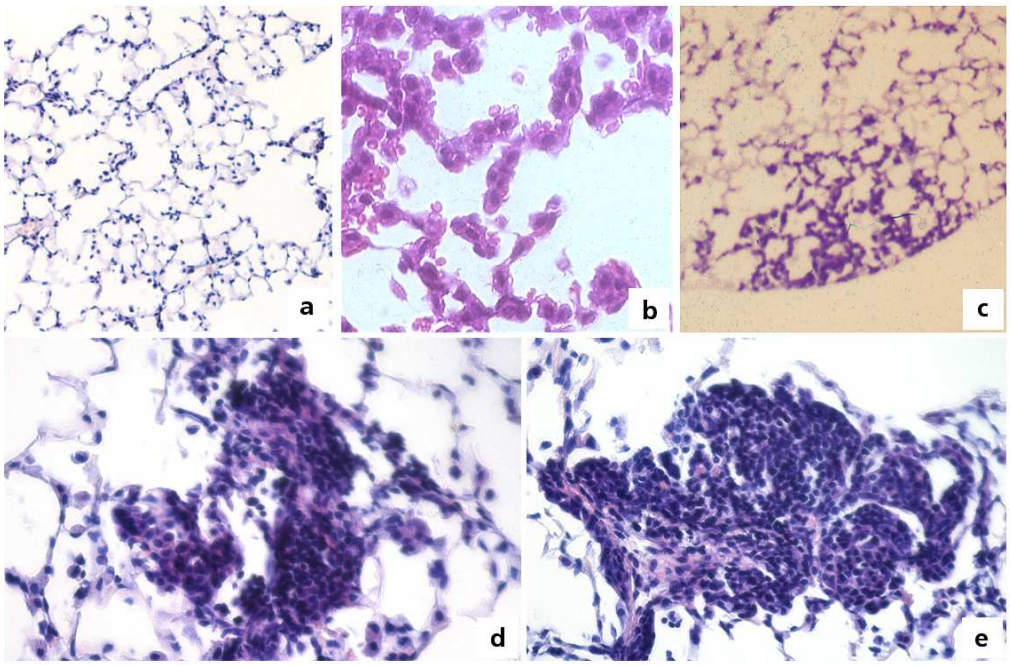


Figure 2

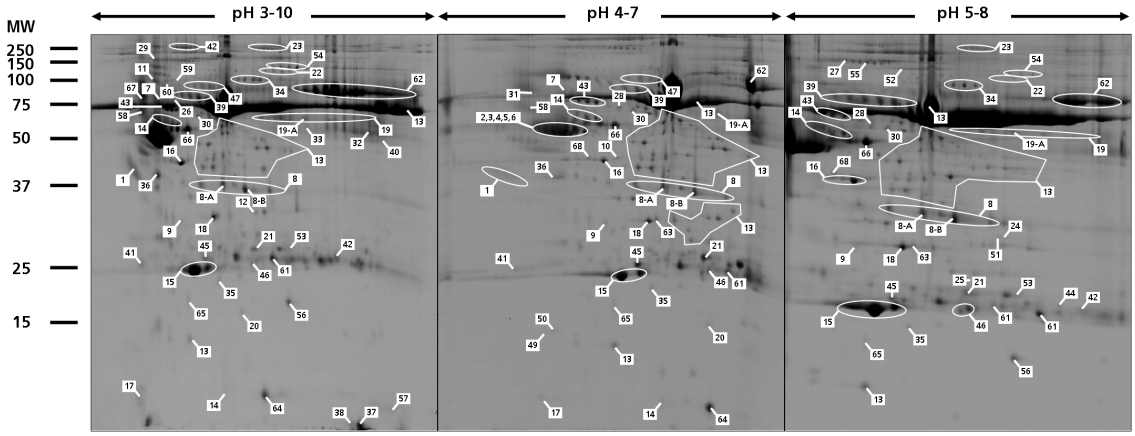


Figure 3

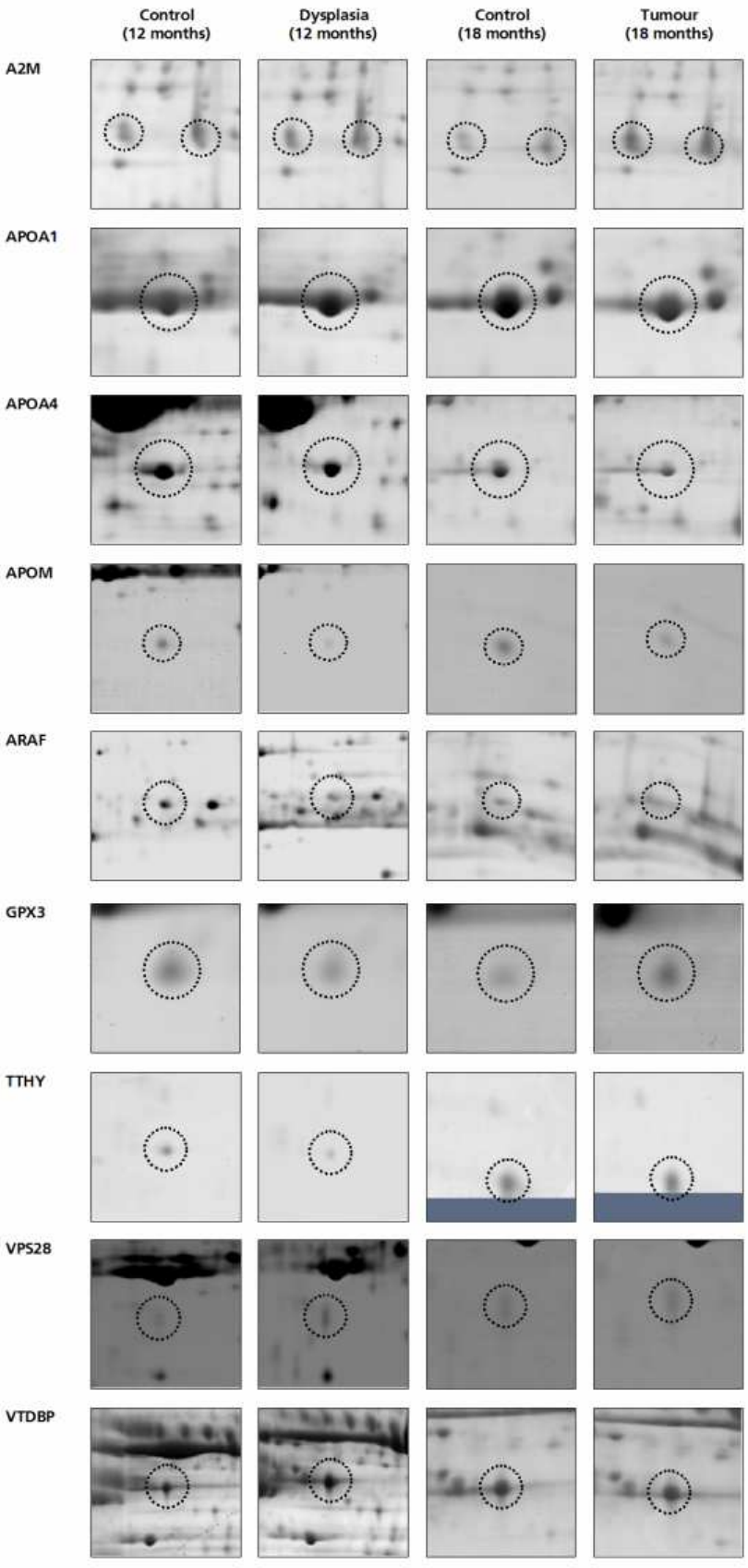

Control (12 months)
Dysplasia (12 months)
Control (18 months)
Tumour (18 months)
A2M
APOA1
APOA4
APOM
ARAF
GPX3
TTHY
VPS28
VTDBP

Figure 4

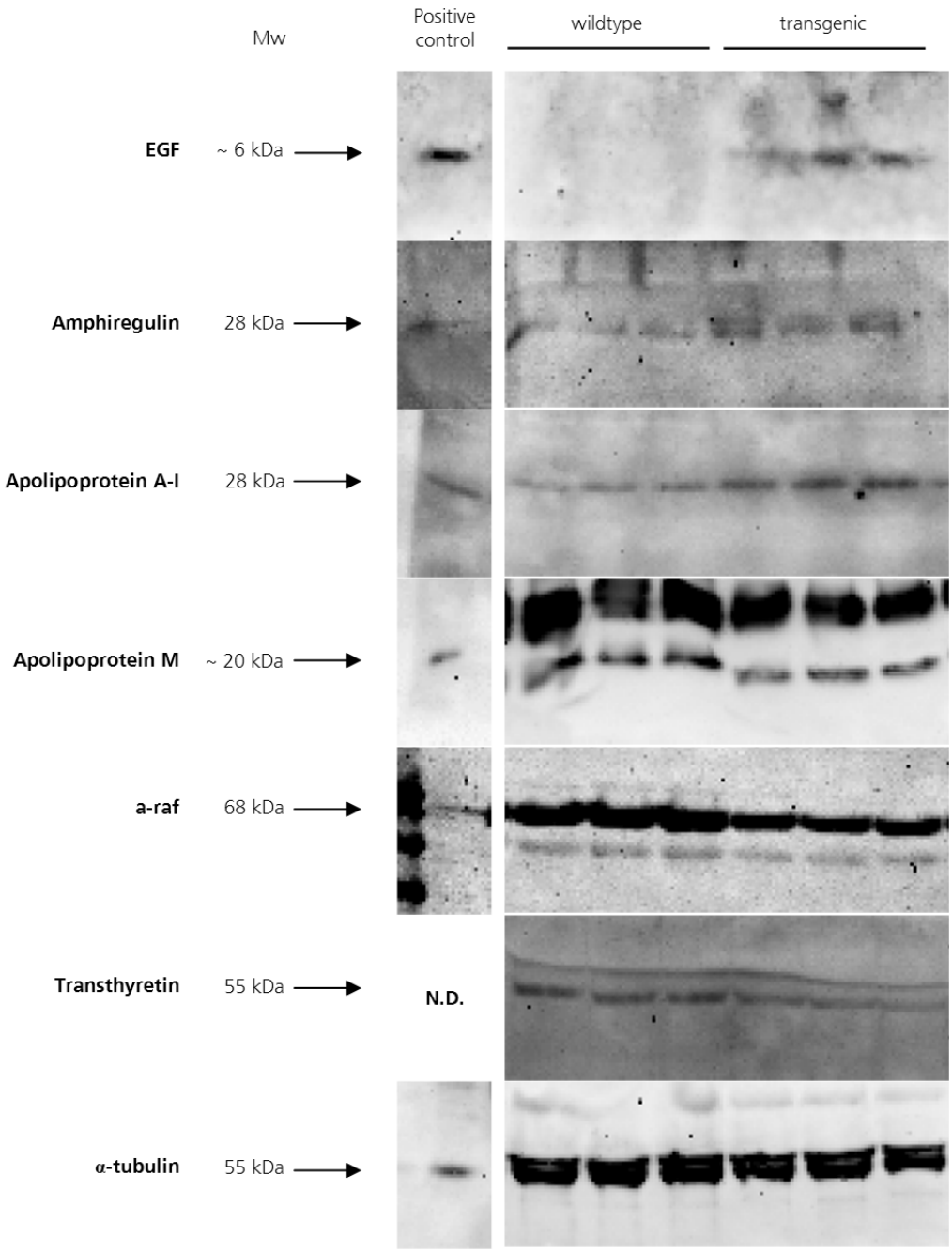
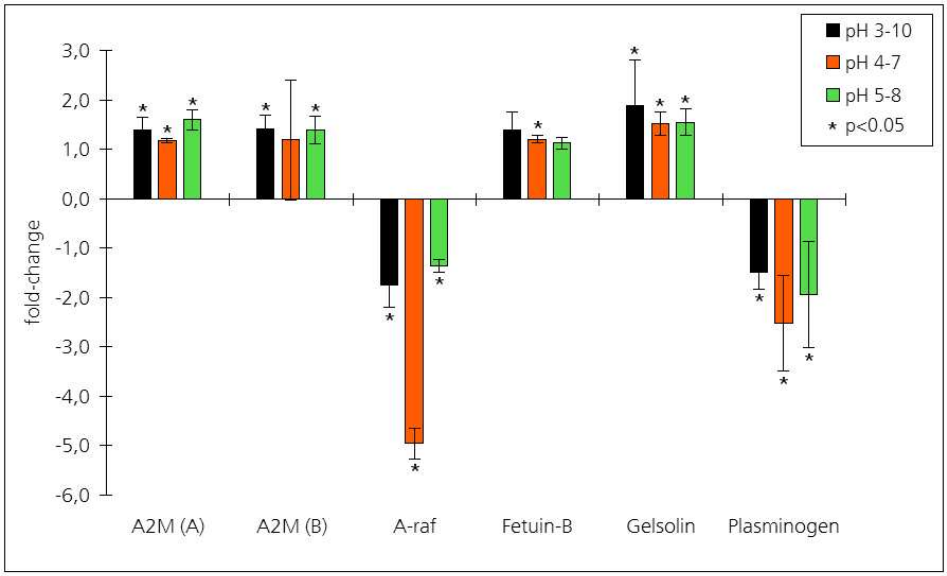
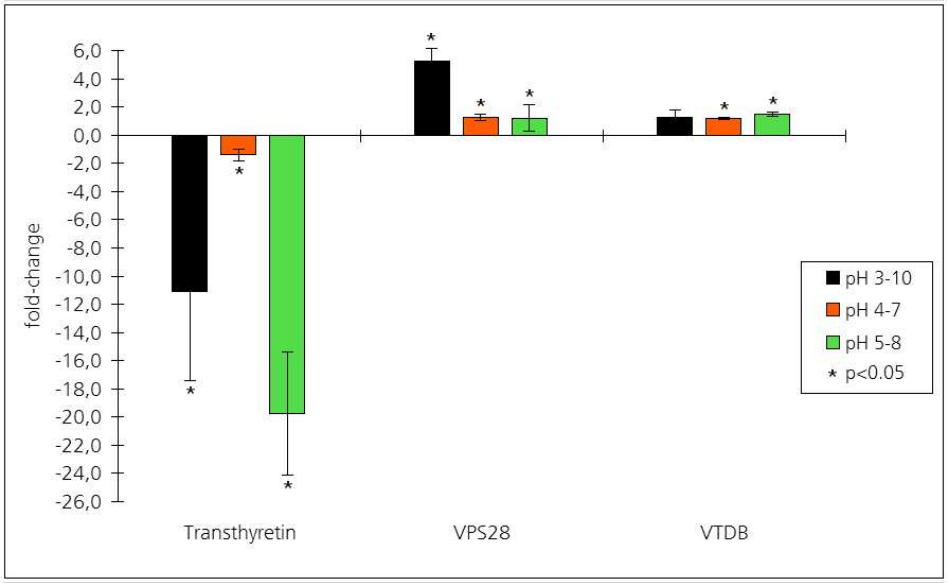

Mw
Positive control
wildtype
transgenic
EGF
~ 6 kDa
Amphiregulin
28 kDa
Apolipoprotein A-I
28 kDa
Apolipoprotein M
~ 20 kDa
a-raf
68 kDa
Transthyretin
55 kDa
N.D.
α-tubulin
55 kDa

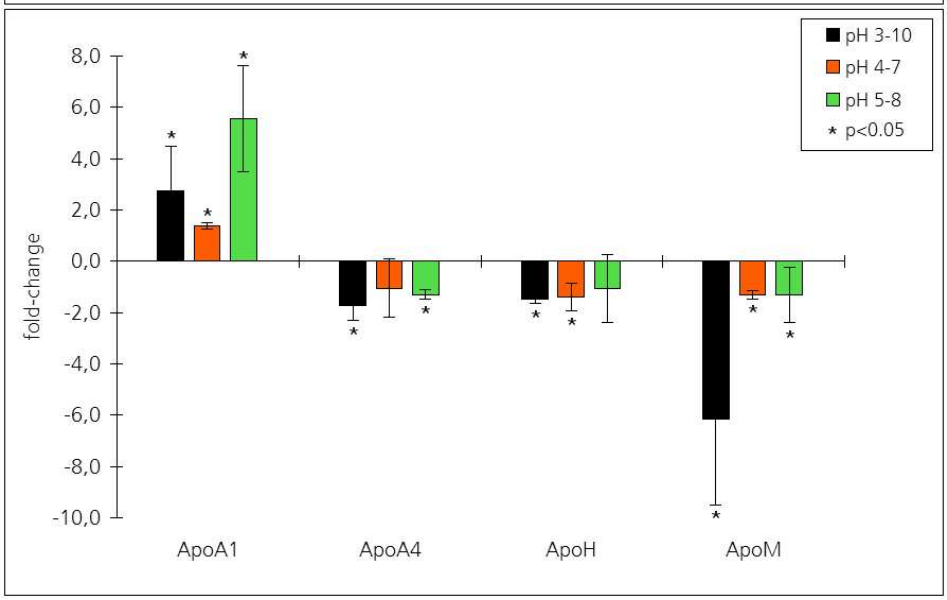
Figure 5a



A



B



C

Figure 5b

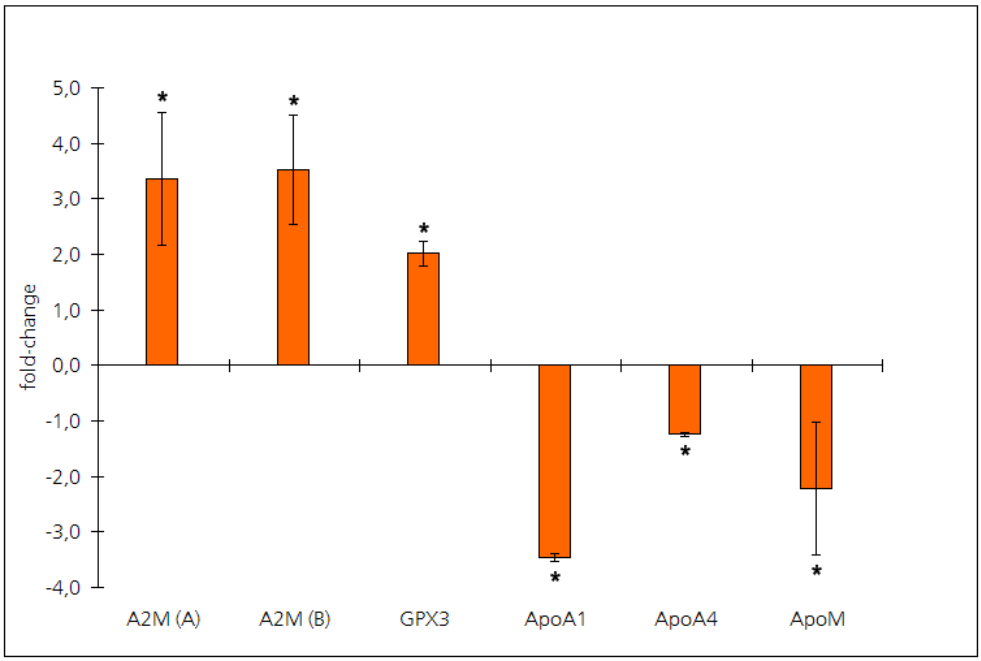

5,0
4,0
3,0
2,0
1,0
0,0
-1,0
-2,0
-3,0
-4,0
fold-change
A2M (A)
A2M (B)
GPX3
ApoA1
ApoA4
ApoM

Figure 6

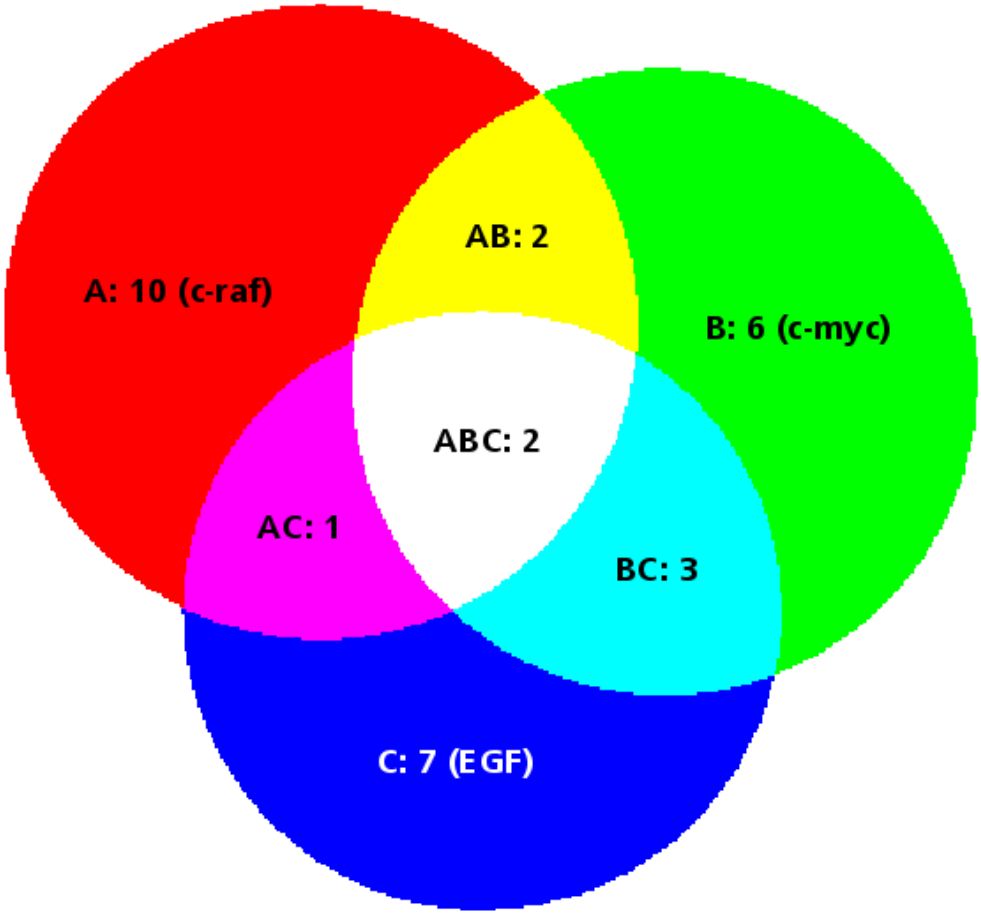


Table 1

No.	Protein-ID	Swiss-Prot Accession #	Protein name
1	A1AG8_MUSCR	P21352	Orosomucoid-8
2	A1AT2_MOUSE	P22599	Alpha-1-antitrypsin 1-2
3	A1AT3_MOUSE	Q00896	Alpha-1-antitrypsin 1-3
4	A1AT4_MOUSE	Q00897	Alpha-1-antitrypsin 1-4
5	A1AT5_MOUSE	Q00898	Alpha-1-antitrypsin 1-5
6	A1AT6_MOUSE	P81105	Alpha-1-antitrypsin 1-6
7	A2AP_MOUSE	Q61247	Alpha-2-antiplasmin
8-A	A2MG_MOUSE	Q61838	Alpha-2-macroglobulin
8-B	A2MG_MOUSE	Q61838	Alpha-2-macroglobulin
9	ACOX3_MOUSE	Q9EPL9	Acyl-coenzyme A oxidase 3, peroxisomal
10	ACTG_MOUSE	P63260	Actin, cytoplasmic 2 (gamma-actin)
11	AFAM_MOUSE	O89020	Afamin
12	AIRE_MOUSE	Q9Z0E3	Autoimmune regulator
13	ALBU_MOUSE	P07724	Serum albumin
14	ANT3_MOUSE	P32261	Antithrombin-III
15	APOA1_MOUSE	Q00623	Apolipoprotein A-I
16	APOA4_MOUSE	P06728	Apolipoprotein A-IV
17	APOC3_MOUSE	P33622	Apolipoprotein C-III
18	APOE_MOUSE	P08226	Apolipoprotein E
19	APOH_MOUSE	Q01339	Apolipoprotein H
19-A	APOH_MOUSE	Q01339	Apolipoprotein H
20	APOM_MOUSE	Q9Z1R3	Apolipoprotein M
21	ARAF_MOUSE	P04627	A-Raf proto-oncogene serine/threonine-protein kinase
22	CFAB_MOUSE	P04186	Complement factor B
23	CFAH_MOUSE	P06909	Complement factor H
24	CFAI_MOUSE	Q61129	Complement factor I
25	CO3_MOUSE	P01027	Complement C3
26	CO9_MOUSE	P06683	Complement component C9
27	COKA1_MOUSE	Q923P0	Collagen alpha-1(XX) chain
28	CPT2_MOUSE	P52825	Carnitine palmitoyltransferase II
29	EGFR_MOUSE	Q01279	Epidermal growth factor receptor
30	FETUB_MOUSE	Q9QXC1	Fetuin-B
31	FGF2_MOUSE	P15655	Heparin-binding growth factor 2
32	GC3_MOUSE	P22436	Immunglobulin gamma-3 chain C region, secreted form
33	GCB_MOUSE	P01866	Immunglobulin gamma-2B chain C region secreted form
34	GELS_MOUSE	P13020	Gelsolin
35	GPX3_MOUSE	P46412	Glutathione peroxidase 3
36	HA10_MOUSE	P01898	H-2 class I histocompatibility antigen, Q10 alpha chain
37	HBA_MOUSE	P01942	Alpha-globin
38	HBB1_MOUSE	P02088	Beta-1-globin
39	HEMO_MOUSE	Q91X72	Hemopexin
40	IGHG1_MOUSE	P01868	Immunglobulin gamma-1 chain C region secreted form
41	IGJ_MOUSE	P01592	Immunglobulin J chain
42	KAC_MOUSE	P01837	Immunglobulin kappa chain C region
43	KNG1_MOUSE	O08677	Kininogen-1
44	KV3B_MOUSE	P01655	Immunglobulin kappa chain V-III region PC 7132
45	KV5L_MOUSE	P01645	Immunglobulin kappa chain V-V region HP 93G7
46	KV6K_MOUSE	P04945	Immunglobulin kappa chain V-VI region NQ2-6.1
47	MUC_MOUSE	P01872	Immunglobulin mu chain C region secreted form
48	MUG1_MOUSE	P28665	Murinoglobin-1
49	MUP2_MOUSE	P11589	Major urinary protein 2
50	MUP8_MOUSE	P04938	Major urinary proteins 11 and 8
51	MYH14_MOUSE	Q6URW6	Myosin-14
52	NAL10_MOUSE	Q8CCN1	NACHT, LRR and PYD domains-containing protein 10
53	PEDF_MOUSE	P97298	Pigment epithelium-derived factor
54	PLMN_MOUSE	P20918	Plasminogen
55	PPCKC_MOUSE	Q9Z2V4	Phosphoenolpyruvate carboxylase
56	RETBP_MOUSE	Q00724	Plasma retinol-binding protein
57	SAA4_MOUSE	P31532	Serum amyloid A-4 protein
58	SPA3K_MOUSE	P07759	Contrapsin
59	TETN_MOUSE	P43025	Tetranectin
60	THRB_MOUSE	P19221	Prothrombin
61	TLN1_MOUSE	P26039	Talin-1
62	TRFE_MOUSE	Q921I1	Serotransferrin
63	TRI30_MOUSE	P15533	Tripartite motif-containing protein 30
64	TTHY_MOUSE	P07309	Transthyretin (Prealbumin)
65	VPS28_MOUSE	Q9D1C8	Vacuolar protein sorting-associated protein 28 homolog
66	VTDB_MOUSE	P21614	Vitamin D-binding protein
67	VTNC_MOUSE	P29788	Vitronectin
68	ZA2G_MOUSE	Q64726	Zinc-alpha-2-glycoprotein

Table 2a

No.	pH	Protein ID	TRANSGENIC								CONTROL								Ratio (T/C)	p
			T1	T2	T3	T4	T5	T6	mean	%RSD	C1	C2	C3	C4	C5	C6	mean	%RSD		
8-A	pH 3-10	A2MG_MOUSE	2980	2514	3039	4099	2714	2556	2984	19.68	2249	2112	1916	2681	2501	1529	2165	19.11	**1.4**	**0.01901**
8-A	pH 4-7	A2MG_MOUSE	5837	5403	5757	6051	5662	5461	5695	4.25	4992	3813	4337	5272	5939	4714	4844	15.28	**1.2**	**0.02326**
8-A	pH 5-8	A2MG_MOUSE	5397	4307	5576	4692	5918	4581	5078	12.59	3353	3274	3617	2628	3047	3166	3181	10.45	**1.6**	**0.00007**
8-B	pH 3-10	A2MG_MOUSE	3021	2986	4833	4145	3544	3274	3634	19.96	2867	2394	2996	2567	2853	1879	2593	15.95	**1.4**	**0.01217**
8-B	pH 4-7	A2MG_MOUSE	4341	4550	5360	6081	6023	6671	5504	16.75	5374	4938	4475	4708	3064	5239	4633	18.06	**1.2**	**0.11718**
8-B	pH 5-8	A2MG_MOUSE	4785	5961	3506	3733	4565	3926	4413	20.47	4053	3172	2744	1947	3452	3727	3182	23.71	**1.4**	**0.02836**
15	pH 3-10	APOA1_MOUSE	1793	1007	1004	1486	4107	3492	2148	61.82	1054	414	1161	583	459	999	778	42.38	**2.8**	**0.03414**
15	pH 4-7	APOA1_MOUSE	2363	2670	2725	2449	2516	2076	2467	9.49	1663	2065	1238	2407	1274	2172	1803	27.03	**1.4**	**0.01325**
15	pH 5-8	APOA1_MOUSE	2503	1439	2103	1057	3037	3006	817	37.28	449	485	589	246	390	203	394	37.26	**5.6**	**0.00035**
16	pH 3-10	APOA4_MOUSE	1488	874	2036	1768	1497	1478	1524	25.39	2402	2079	3036	2866	2243	3007	2606	15.96	**0.6**	**0.00089**
16	pH 4-7	APOA4_MOUSE	2425	2123	2040	2526	2846	2646	2434	12.66	2823	2602	2600	2859	2405	2094	2564	11.07	**0.9**	**0.46633**
16	pH 5-8	APOA4_MOUSE	2285	2026	1984	1701	2168	1618	1964	13.24	2264	2588	2628	3007	2303	2544	2556	10.49	**0.8**	**0.00305**
19	pH 3-10	APOH_MOUSE	821	950	1049	794	919	1004	923	10.86	1461	788	1715	817	1526	1784	1349	32.57	**0.7**	**0.04320**
19	pH 4-7	APOH_MOUSE	1284	1662	1056	1201	1647	709	1260	28.85	2295	1712	1847	1890	1311	1567	1770	18.76	**0.7**	**0.02936**
19	pH 5-8	APOH_MOUSE	807	453	782	814	966	701	754	22.63	956	919	615	362	1031	898	797	32.10	**0.9**	**0.73822**
20	pH 3-10	APOM_MOUSE	160	102	224	155	432	219	215	53.59	431	508	1582	2321	1882	1222	1324	56.96	**0.2**	**0.00519**
20	pH 4-7	APOM_MOUSE	344	263	262	341	259	311	297	13.64	307	349	414	400	496	362	388	16.80	**0.8**	**0.01526**
20	pH 5-8	APOM_MOUSE	138	127	155	179	223	252	179	27.72	208	240	209	249	242	241	232	7.88	**0.8**	**0.03515**
21	pH 3-10	ARAF_MOUSE	904	1302	1638	1204	1632	1503	1364	20.93	3280	2011	1525	3333	1343	2876	2395	36.91	**0.6**	**0.02161**
21	pH 4-7	ARAF_MOUSE	2153	2207	3012	2630	2973	3275	2708	16.92	3539	2749	3364	2483	2450	3190	2963	15.71	**0.9**	**0.36259**
21	pH 5-8	ARAF_MOUSE	2162	2164	1985	2367	2131	2654	2244	10.48	2603	3572	2830	2646	2975	3709	3056	15.52	**0.7**	**0.00373**
30	pH 3-10	FETUB_MOUSE	407	365	370	334	582	286	391	26.14	437	174	337	305	283	154	282	37.45	**1.4**	**0.09898**
30	pH 4-7	FETUB_MOUSE	611	588	636	637	558	555	598	6.08	544	423	470	405	512	633	498	16.93	**1.2**	**0.02353**
30	pH 5-8	FETUB_MOUSE	569	516	444	490	453	432	484	10.73	447	410	413	431	408	479	431	6.44	**1.1**	**0.05312**
34	pH 3-10	GELS_MOUSE	2133	1705	590	956	727	1413	1254	47.96	772	374	1024	725	575	504	662	34.60	**1.9**	**0.04804**
34	pH 4-7	GELS_MOUSE	1140	1181	986	1299	1541	1152	1216	15.44	755	953	783	804	847	669	802	11.85	**1.5**	**0.00070**
34	pH 5-8	GELS_MOUSE	703	766	745	561	522	823	687	17.40	410	638	433	374	465	348	445	23.30	**1.5**	**0.00378**
35	pH 3-10	GPX3_MOUSE	457	159	206	272	147	86	221	59.36	327	317	838	1221	751	419	646	55.41	**0.3**	**0.02124**
35	pH 4-7	GPX3_MOUSE	867	372	461	431	339	268	456	46.59	420	182	227	500	537	526	399	39.19	**1.1**	**0.60568**
35	pH 5-8	GPX3_MOUSE	709	248	298	289	243	183	328	58.13	227	267	269	186	301	365	269	22.97	**1.2**	**0.48609**
54	pH 3-10	PLMN_MOUSE	574	372	728	474	497	521	528	22.48	1131	558	479	758	817	964	785	31.15	**0.7**	**0.04309**
54	pH 4-7	PLMN_MOUSE	695	421	573	666	523	296	529	28.55	1090	1506	1826	1330	1350	905	1334	24.06	**0.4**	**0.00024**
54	pH 5-8	PLMN_MOUSE	172	163	201	117	73	69	132	41.50	177	250	136	252	253	470	256	44.96	**0.5**	**0.03864**
64	pH 3-10	TTHY_MOUSE	75	372	33	28	110	29	108	123.76	1657	1022	1706	829	950	992	1193	32.25	**0.1**	**0.00007**
64	pH 4-7	TTHY_MOUSE	319	409	247	386	495	487	391	24.64	405	605	587	478	709	471	543	20.49	**0.7**	**0.02990**
64	pH 5-8	TTHY_MOUSE	55	18	240	297	519	303	239	76.89	4750	4759	6444	5860	4076	2356	4708	30.45	**0.1**	**0.00002**
65	pH 3-10	VPS28_MOUSE	1459	1787	1854	1334	1283	1317	1506	16.73	262	260	312	432	202	243	285	28.12	**5.3**	**5.09E-07**
65	pH 4-7	VPS28_MOUSE	288	283	397	412	352	292	337	17.22	281	242	248	294	286	230	264	10.16	**1.3**	**0.01816**
65	pH 5-8	VPS28_MOUSE	562	373	436	349	466	406	432	17.66	388	390	363	317	341	328	355	8.66	**1.2**	**0.04417**
66	pH 3-10	VTDB_MOUSE	4933	3755	7248	5305	3082	2322	4441	39.87	2442	2964	4542	3520	5883	1322	3446	46.61	**1.3**	**0.33182**
66	pH 4-7	VTDB_MOUSE	6453	5409	6410	5686	6370	5706	6006	7.61	5056	5381	4905	5860	4733	4058	4999	12.19	**1.2**	**0.00889**
66	pH 5-8	VTDB_MOUSE	6000	7108	6638	5759	6009	5289	6134	10.54	5788	6212	2937	4616	2855	2603	4169	38.22	**1.5**	**0.01882**

Table 2b

No.	Protein ID	TRANSGENIC						CONTROL								Ratio (T/C)	p
		T1	T2	T3	T4	mean	%RSD	C1	C2	C3	C4	C5	C6	mean	%RSD		
8-A	A2MG_MOUSE	1290	556	1155	784	946	35.6	160	406	147	137	415	421	281	51.94	**3.4**	**0.00241**
8-B	A2MG_MOUSE	2869	1603	3172	2241	2471	28.19	527	1282	280	253	1021	844	701	59.44	**3.5**	**0.00094**
15	APOA1_MOUSE	1234	1266	1250	1294	1261	2.03	4151	4053	4792	4079	4447	4746	4378	7.63	**0.3**	**8.36E-08**
16	APOA4_MOUSE	1340	1344	1266	1315	1316	2.73	1520	1735	1715	1477	1506	1877	1638	9.83	**0.8**	**0.00480**
19	APOH_MOUSE	969	787	636	872	816	17.3	1337	1318	142	979	861	310	825	60.93	**1.0**	**0.97334**
20	APOM_MOUSE	126	74	122	49	93	40.47	239	96	166	216	312	206	206	35.12	**0.5**	**0.02167**
21	ARAF_MOUSE	2676	4930	2116	1433	2789	54.34	1299	7361	2143	1873	3800	2183	3110	72.12	**0.9**	**0.80997**
30	FETUB_MOUSE	511	406	356	557	458	20.24	342	654	504	206	592	428	454	36.34	**1.0**	**0.97334**
35	GPX3_MOUSE	414	410	463	355	411	10.76	218	144	283	131	253	195	204	29.29	**2.0**	**0.00037**
64	TTHY_MOUSE	2845	3807	3258	2780	3173	14.91	3822	4897	2733	4051	2487	N.D.	3598	27.53	**0.9**	**0.45943**
65	VPS28_MOUSE	392	170	229	115	227	52.87	94	130	115	56	141	282	136	56.80	**1.7**	**0.18181**
66	VTDB_MOUSE	4049	3882	3372	3698	3750	7.73	3766	3792	3327	2709	3503	4552	3608	16.85	**1.0**	**0.67885**

Table 2c

Protein	Lung dysplasia	Lung cancer
α-2 macroglobulin	1.6 ↑	3.5 ↑
Apolipoprotein A-I	5.6 ↑	3.5 ↓
Apolipoprotein A-IV	1.7 ↓	1.2 ↓
Apolipoprotein H	1.5 ↓	1.0 ↔
Apolipoprotein M	6.2 ↓	2.2 ↓
A-raf	1.8 ↓	1.1 ↓
Fetuin B	1.4 ↑	1.0 ↔
Gelsolin	1.9 ↑	N.D.
Glutathione peroxidase 3	2.9 ↓	2.0 ↑
Plasminogen	2.5 ↓	N.D.
Transthyretin	19.7 ↓	1.1 ↓
Vacuolar protein sorting-associated protein 28 homolog	5.3 ↑	1.7 ↑
Vitamin D binding protein	1.5 ↑	1.0 ↔

Supplementary Table 1:
A summary of serum proteins from SP-C/IgEGF transgenic mice

ssprot ID	Swissprot Accession no.	Protein name and aliases	Mascot score (PMF) No. of matched peptides Percentual coverage	Peptide sequences from PMF	Ions score (PFF) No. of matched peptides Percentual coverage	Peptide sequences from PFF	Parent mass	Gene and synonyms	Mr (Da) pI	Keywords
AGB_MUSCR	P21352	Alpha-1-acid glycoprotein B [Precursor] AGP B Orosomucoid-B OMD B	-	-	72 1 7	YEGGVIETFAHLMLR	17338.8719	Orm8 Agp-8 Orm-8	24311 5.58	Acute phase; Glycoprotein; Secreted; Signal
AT2_MOUSE	P22599	Alpha-1-antitrypsin 1-2 [Precursor] Serine protease inhibitor 1-2 Alpha-1 protease inhibitor 2 Alpha-1-antiproteinase AAT	147 13 53	LVQHIPR TLMSPLGITR + OxPM RLVQHIPR ELISKLLIHR ELDQDTVFALAIHYLFK IFNNIGADLSGITEENAPLK NHYQAEVFSVNFAESEEAKK WKKPFDPEHTEEAEFHVDK DQSPASHEIATNILGDFALSLYR FDHIFLFIFEEHTQSPIFVGK AVLTIDETGTEAAAATVFEAVPMSAPPPILR + 2 OxPM SFQHLLQTLNRPDSELQLSTGNGLFVNHDLK	-	-	-	Orm8 Agp-8 Orm-8	24311 5.58	Acute phase; Glycoprotein; Secreted; Signal
AT3_MOUSE	P07758	Alpha-1-antitrypsin 1-3 [Precursor] Serine protease inhibitor 1-3 Alpha-1 protease inhibitor 3 Alpha-1-antiproteinase AAT	-	-	126 2 7	DQSPASHEIATNILGDFAISLYR LAQHIPFR	2435.1819 981.5453	Serpina1c Dom3 Spi1-3	45996 5.32	Acute phase; Glycoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal
AT4_MOUSE	Q03897	Alpha-1-antitrypsin 1-4 [Precursor] Serine protease inhibitor 1-4 Alpha-1 protease inhibitor 4 Alpha-1-antiproteinase AAT	134 10 41	SDAQHIPR TLMSPLGITR TLMSPLGITR + OxPM ELISQFLLHR DQSPASHEIATNILGDFALR NHYQAEVFSVNFAESEEAKK FDHIFLFIFEEHTQSPIFVGK TLMSPLGITRFINNGADLSGITEENAPLK + OxPM AVLTIDETGTEAAAATVLQVATYSAPPRVIR + OxPM SFQHLLQTLNRPDSELQLSTGNGLFVNHDLK	-	-	-	Serpina1d Dom4 Spi1-4	46143 5.24	Glycoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal
AT5_MOUSE	Q03898	Alpha-1-antitrypsin 1-5 [Precursor] Serine protease inhibitor 1-5 Alpha-1 protease inhibitor 5 Alpha-1-antiproteinase AAT	-	-	78 2 7	ELISKFLLHR DQSPASHEIATNILGDFAISLYR	1232.6723 2435.1253	Serpina1e Dom5 Spi1-5	46391 5.44	Glycoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal
AT6_MOUSE	P81135	Alpha-1-antitrypsin 1-6 [Precursor] Serine protease inhibitor 1-6 Alpha-1 protease inhibitor 6	151 14 42	LAQHIPR TLMSPLGITR TLMSPLGITR + OxPM RLAQHIPR MQHLEQTLSK ELISKFLLHR IFNNIGADLSGITEENAPLK NHYQAEVFSVNFAESEEAK NHYQAEVFSVNFAESEEAKK DQSPASHEIATNILGDFAISLYR KPEDPEHTEEAEFHVDESTTVK FDHIFLFIFEEHTQSPLFVGK TLMSPLGITRFINNGADLSGITEENAPLK + OxPM SFQHLLQTLNRPDSELQLSTGNGLFVNHDLK	-	-	-	Spi1-6 Dom6	45966 5.25	Glycoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal
AP_MOUSE	Q61247	Protein name: Alpha-2-antiplasmin [Precursor] Alpha-2-AP Alpha-2-plasmin inhibitor Alpha-2-PI	66 11 26	LPLPALFK DFTHLDER NPNIPSALPQLQEQR GFPIKDDFLEQSER FTVSYDMVMHAVSYRLR FTVSYDMVMHAVSYRLR + OxPM FTVSYDMVMHAVSYRLR + 2 OxPM WFLLEQPEIQVAHFPFK VLHLMNTGSCLPHLLSHFYQHLGPQTIR VLHLMNTGSCLPHLLSHFYQHLGPQTIR + OxPM LHLQQQLDLVATLSQLGLQELFQGPDLR	-	-	-	Serpinf2 Pli	55185 5.85	3D-structure; Acute phase; Direct protein sequencing; Glycoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal; Sulfation
MG_MOUSE	Q61838	Alpha-2-macroglobulin [Precursor] Alpha-2-M Pregnancy zone protein	-	-	117 2 1	TFHVYISQHR APFALQVNITLPLNFDK	1231.5343 1787.9723	A2m Pzp	167391 6.27	Bait region; Direct protein sequencing; Glycoprotein; Phosphoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal; Thioester bond
OX3_MOUSE	O9EPL9	Peroxisomal acyl-coenzyme A oxidase 3 EC 1.3.3.6 Pristanoyl-CoA oxidase Branched-chain acyl-CoA oxidase BRCACox	-	-	29 1 1	LAVSIAIR	842.4523	Acox3	79233 7.34	FAD; Fatty acid metabolism; Flavoprotein; Lipid metabolism; Oxidoreductase; Peroxisome
TG_MOUSE	P63260	Actin, cytoplasmic 2 Gamma-actin	60 6 18	AGFAGDDAPR GYSFTTTAER AYPSYQRPR MVHHTFYNELR QEYDESGPSIVHR SYELPDGQVITIGNER	-	-	-	Actg1 Actg	42138 5.31	Acetylation; ATP-binding; Cytoplasm; Cytoskeleton; Direct protein sequencing; Methylation; Nitration; Nucleotide-binding; Phosphoprotein; Structural protein
4M_MOUSE	O89023	Afamin [Precursor] Alpha-albumin Alpha-Alb	78 12 23	CQAYKNK ENPAGCYR RLCFFYNK RTESWVCQER FFAEFIYEVSR VYMDFLEDCCSR VYMDFLEDCCSR + OxPM ANVGFLPPFFLDPEEK DSDPDLPFAEFIYEYSR EACERAAHNDDRPECLSLR WVMPGITDEWVLCLFTKTAAR DLTTLLEDVSSMVEQCCEQDVVHCIR + OxPM	-	-	-	Afm	71529 5.54	Alternative splicing; Glycoprotein; Repeat; Secreted; Signal; Transport
IE_MOUSE	Q923E3	Autoimmune regulator Synonyms:Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy protein homolog APECED protein homolog	53 5 11	MAGDDGMLLR + OxPM ATPPATLASK DYNLERVSR SVSSPGSHLKTKPPK GAVEGLEQQVTESGRSK	-	-	-	Aire	60146 8.48	Alternative splicing; Cytoplasm; DNA-binding; Metal-binding; Nucleus; Phosphoprotein; Repeat; Transcription; Transcription regulation; Zinc; Zinc-finger
BU_MOUSE	P07724	Serum albumin [Precursor]	174 13 26	LCAAPILR YNGVFQECHPK NPDVSVSLLLR APQVSTPTLVEAAR RHPDYSVSLLLR LGEYGFQNALIVR DVFLGTFLYEYSR LPCVEDYLSAILNR LSQTFRNADFAEITK QEPERNECFLQHK RPCFSALTVDETYVPK EFPTTMSGYVLPEVAR + OxPM AADKDTCFSTEGPNLVTR	53 1 2	LGEYGFQNALIVR	1479.8313	Alb Alb1 Alb-1	73733 5.75	Cleavage on pair of basic residues; Copper; Direct protein sequencing; Lipid-binding; Metal-binding; Phosphoprotein; Repeat; Secreted; Signal
F3_MOUSE	P32261	Antithrombin-III [Precursor] ATIII	127 18 39	FSPEINTR ENPEQSR LQPLDFK TEDGFSLK RVWELSK SQLPGVAGGR AIIRPFLVLIR TSDQHFFFAK DIPMNPLCIYR EVALIHTIFMGR EVALIHTIFMGR + OxPM FATHFVQHLADSK RVAEGTQVLELPFK EQLQDMGLDLFSPEK EQLQDMGLDLFSPEK + OxPM AFLEVNEEGSEAAASTSVVITGR SQLPGVAGGRDDLYYSDAFHK VAEGTQVLELPFKGDITMVLILPKPEK	52 2 4	DIPMNPLCIYR AIIRPFLVLIR	1359.7143 1198.7463	Serpinc1 At3	52484 6.13	Blood coagulation; Glycoprotein; Heparin-binding; Protease inhibitor; Secreted; Serine protease inhibitor; Signal
OA1_MOUSE	Q03623	Apolipoprotein A-I [Precursor] Apo-AI ApoA-I	186 15 41	ETDVVR THVDSLR LQELQGR EDVELYR ARPALEDLR LSPVAEEFR QKLQELQGR WLEDVELYR VQPYLDDFQK TQLAPHSEQMR + OxPM LSPVAEEFRDR SWFLLIEYNTR VAPLGAELQESAR DFWDNLEKETDWVR LAELKSNFLNEYHER	27 1 4	VAPLGAELQESAR	1340.7393	Apoa1	93569 5.64	Cholesterol metabolism; HDL; Lipid metabolism; Lipid transport; Repeat; Secreted; Signal; Steroid metabolism; Transport

0A4_MOUSE	P36728	Apolipoprotein A-IV [Precursor] Apo-AIV ApoA-IV	96 11 34	GNTEGLQK LQLTPVQR ALVQQLEQFR QLEQQVEEFR ATIDQNLEDLRR QLEQQVEEFRR LNHQMEGLAFQMK + 2 OxiM TDVTQQLSTLFQDK LVPFVVQLSGHLAKETER QQLQPYSQEVESHLSPLEK LQEHLKPVAVDLQDQINTQTQEMK + OxiM	135 1 4	LVPFVVQLSGHLAKETER	2323.2483	Apoa4	45331 5.41	Chylomicron; HDL; Lipid transport; Polymorphism; Repeat; Secreted; Signal; Transport
OC3_MOUSE	P33622	Apolipoprotein C-III [Precursor] Apo-CIII ApoC-III	-	-	128 2 27	GWVADHMFR + OxiM TVQDALSSVQESDIAVVAR	1078.4313 1987.9913	Apoc3	10975 4.64	Chylomicron; Lipid degradation; Lipid transport; Secreted; Signal; Transport; VLDL
OE_MOUSE	P38226	Apolipoprotein E [Precursor] Apo-E	144 15 41	EVQAAQAR FWDYLR ORLEEVR LGPLVEQGR LQADVMEDLR + OxiM LQAEIFQAR GRLEEVGNQAR TANLGAGAAQPLR SKMEEQTQQR + OxiM TANLGAGAAQPLRDR GWVEERVEDMHR GWFERVEDMHR + OxiM ELEEQLGPVAEETR NEVHTMLGQSTEEIR + OxiM LKGWVEERVEDMHR + OxiM	32 1 2	LQAEIFQAR	1375.5873	Apoe	35931 5.56	3D-structure; Chylomicron; Direct protein sequencing; HDL; Heparin-binding; Lipid transport; Repeat; Secreted; Signal; Transport; VLDL
OH_MOUSE	Q31339	Beta-2-glycoprotein 1 [Precursor] Beta-2-glycoprotein I Apolipoprotein H Apo-H B2GPI Beta(2)GPI Activated protein C-binding protein APC inhibitor	152 15 19	ATVLYQGMR + OxiM IFCPPPVPK WVSPDPACAR DGTEIPSCFK CSYTVEAHCR TGFWVSFLPTCR KCSYTVEAHCR VCPFAGILENGVR IQEQFKNGMMHGDK FTCRLTGMVPNTLR FTCRLTGMVPNTLR + OxiM CTEEGKWSPDIPACAR KCPKPDDLPFATVVPLK TSYDPQEQVYSCKPGYVSR CPFPRPENGYVNYPAKPVLLYK	75 1 4	VCPFAGILENGVR	1544.8233	Apoh B2gp1	39934 8.59	Glycoprotein; Heparin-binding; Repeat; Secreted; Signal; Sushi
OM_MOUSE	Q9Z1R3	Apolipoprotein M Apo-M ApoM	46 3 11	AFLVTPR FLLYNR ETGQGYQR	-	-	-	Apom Itg23	21632 6.38	HDL; Lipid transport; Secreted; Signal; Transport
AF_MOUSE	P34627	A-Raf proto-oncogene serine/threonine-protein kinase EC 2.7.11.1	-	-	32 1 1	SPHSKLPSEQR	1265.6513	Araf A-raf Araf1	68223 9.27	ATP-binding; Kinase; Metal-binding; Nucleotide-binding; Phorbol-ester binding; Phosphoprotein; Proto-oncogene; Serine/threonine-protein kinase; Transferase; Zinc; Zinc-finger
4B_MOUSE	P34186	Complement factor B [Precursor] EC 3.4.21.47 C3/C5 convertase	139 12 25	TCQENGR VASYSYPPR QQLVPSYAR RQQLVPSYAR LEDVTYHCSR FQVQGVSVSVQYVDVCR CPRPQDFENGEFVCPR YQQTLRPICIPCTEGTTR WDGQTAICDDGSAGYCPNPGIPIGTR HVIIMTDGLHNMGGNPVTVIQDIR + 2 OxiM QGSFQLLQEGQALEYLCPSGFYPVPVQTR GHETCMGAVVSEVFVLTAAHCFMVDDQK + 2 OxiM	-	-	-	Cfb Bf H2-Bf	86319 7.18	Cleavage on pair of basic residues; Complement alternate pathway; Glycoprotein; Hydrolase; Immune response; Innate immunity; Protease; Repeat; Secreted; Serine protease; Signal; Sushi; Zymogen
4H_MOUSE	P36939	Complement factor H [Precursor] Protein beta-1-H	152 21 25	WVASNPSR TQEQVTFR VKGQPVCK CVEILCTPPR IHHSDDEIR CTPTGWPVPR CLPVTELENGR CTLKPCEFPQFK VQDLLEFSCHSGHR KPCGHPGDTPFGSFR CTAQGWEPEVPCVR CSPPYLNGIYTPHR IPCSQPPTIEHGSYKLPR CDNGFSPPSGYSVVDYLR LVYEESLRPWFPSKHK IVSGAATDQDEYYFGQVVR YVYTCDDGYQLLGEDVR GSFCTVYGAGDTPSCYER DSIESSHEHGTTFSYVCDDGFR SDDEIRVECNYGFYPTGSTVSK TCSASDIHIDNGFLSESSSYALHR	-	-	-	Cfh Hf1	143667 6.63	Complement alternate pathway; Glycoprotein; Immune response; Innate immunity; Repeat; Secreted; Signal; Sushi
4I_MOUSE	Q61129	Complement factor I [Precursor] EC 3.4.21.45 C3B/C4B inactivator	-	-	36 1 1	VAIYYFDWESYHVGR	1726.8193	If Cfi	69471 7.36	Cleavage on pair of basic residues; Complement pathway; Glycoprotein; Hydrolase; Immune response; Innate immunity; Protease; Repeat; Secreted; Serine protease; Signal
3_MOUSE	P31327	Complement C3 [Precursor] Synonym:HSE-MSF	59 11 8	LPYSVVR VNWIFLK AVLFNVR NEQVEIR GICVADVEIR VMQDFTIDLR + OxiM AAYFINHFSDGIK IPYYFYLVMNK + OxiM KVLMEGVRPSNADALVGK SILEEDIIEEDIKER AGEYIEASYMFILQRPVTVAIAGYALALMNK + 2 OxiM	-	-	-	C3	187934 6.39	Alternative initiation; Cleavage on pair of basic residues; Complement alternate pathway; Complement pathway; Direct protein sequencing; Glycoprotein; Immune response; Inflammatory response; Innate immunity; Phosphoprotein; Secreted; Signal; Thioester bond
9_MOUSE	P36683	Complement component C9 [Precursor]	64 7 23	DNIIDDVTSFIR TPFDNEFYNGLCDR EEQEQHYPIPDCR DASVTASVNADCIKTDNGK TDFAINWASSLANAPALLSQR VAEESELGLTAGYGIRILGLMEPLR + OxiM GSEPTQECEEQECEEQFKCGNDFQCETGR	-	-	-	C9	63217 5.56	Complement alternate pathway; Complement pathway; Cytolysis; EGF-like domain; Glycoprotein; Immune response; Innate immunity; Membrane; Membrane attack complex; Secreted; Signal; Transmembrane
KA1_MOUSE	Q923P3	Collagen alpha-1(XX) chain [Fragment]	64 7 13	TGLQGPKGMR + OxiM DLMSATKVR + OxiM LQEVQTPESLE SEVPQTRSHAVLR HVEYEIFILAYYR IFTGSFHKVHAAVGHSK LNTLEHEIARPPMPMAETAKLGRPR	-	-	-	Col23a1	81368 9.3	Alternative splicing; Collagen; Glycoprotein; Repeat; Secreted
T2_MOUSE	P52825	Carnitine O-palmitoyltransferase 2, mitochondrial [Precursor] EC 2.3.1.21 Carnitine palmitoyltransferase II CPT II	55 5 13	EFLHCVQK LSSALKAGVTAAK TEVLCKDFENGIGK VLSAQRLRLLHDEQFR DSVVLNFNPFMAFNPDPK + OxiM	-	-	-	Cpt2 Cpt-2	74538 8.46	Acetylation; Acyltransferase; Fatty acid metabolism; Lipid metabolism; Membrane; Mitochondrion; Mitochondrion inner membrane; Transferase; Transit peptide; Transport
FR_MOUSE	Q01279	Epidermal growth factor receptor [Precursor] EC 2.7.10.1	62 11 11	CHILEGEPR IPLENLQIIR NLQEILGAVR ILCAQQCSGR NYVVTDHGSCVR VCTASGDLHFLPGAFK TIQEVAGYVLIALNTVER FSNNPLCNVDTIQWR FSNNPLCNVDTIQWR + OxiM SPSDCCHNQCAAGCTGPR AVNHMCNPLCSSEGCWGPEPR	-	-	-	Egfr	138187 6.46	ATP-binding; Glycoprotein; Kinase; Membrane; Nucleotide-binding; Phosphoprotein; Receptor; Repeat; Signal; Transferase; Transmembrane; Tyrosine-protein kinase; Ubl conjugation
UB_MOUSE	Q9QXC1	Fetuin-B [Precursor] IRL685	65 8 25	LVVLPFPGK DGYMLSLNR + OxiM AMFHNNKPR AMFHNNKPR + OxiM AMNQWVSPAYVEYVLK + OxiM SQASCSLQHSDSEPVGICQGSTVQSSLR EHYQEDMGSLFVLTLDVLETDCHVLSR EHYQEDMGSLFVLTLDVLETDCHVLSR + OxiM	-	-	-	Fetub	43541 6.17	Glycoprotein; Repeat; Secreted; Signal

F2_MOUSE	P15655	Heparin-binding growth factor 2 [Precursor] HBGF-2 Basic fibroblast growth factor BFGF Prostatropin	55 6 49	TGQYKLGSK YLALKEDGR + OxPM NGGFFLRIHPDGR TGPGQKAILFLPMSAK + OxPM MKAGSITSLPALPEDGGAAFPPGHFK AASGITSILPALPEDGGAAFPPGHFKDPK	-	-	-	Fgf2 Fgf-2	17373 9.58	Angiogenesis; Developmental protein; Differentiation; Growth factor; Heparin-binding; Mitogen
3_MOUSE	P22436	Ig gamma-3 chain C region	53 5 17	ALPAPIER WHYGALSSGVR EVNTAAVTQPR AQTPQVYTIPPPR VVSALPIQHQDWMSR + OxPM	-	-	-	-	36662 8.53	3D-structure; Alternative splicing; Cell membrane; Glycoprotein; Immunoglobulin C region; Immunoglobulin domain; Membrane; Secreted; Transmembrane
2B_MOUSE	P01866	Ig gamma-2B chain C region	68 8 39	DLPSPIER TDSFSCIVR APQVYILPPPAEQLSR VTCVVVDVSEDDPDVR VVSALPIQHQDWMSGK + OxPM CPAPILEGGPSVFIFPPRIIK ISWFVNNVEVHTAQTQTHR TTPPSVYPLAPGCGDTTGSSVTLGCLVK	72 1 4	APQVYILPPPAEQLSR	1778.9659	Igh-3	37244 7.19	3D-structure; Alternative splicing; Cell membrane; Glycoprotein; Immunoglobulin C region; Immunoglobulin domain; Membrane; Repeat; Secreted; Transmembrane
L5_MOUSE	P13020	Gelsolin [Precursor] Actin-depolymerizing factor ADF Brevin	121 16 27	LFACSNR IEPEFVVR EPGLQWR DGGQTAPASIR AGKEPGLQWVR HVVPNEVVVQR SEDCFILDHGR EVQGFESTFSGVFK QTQVSVLPEGGETPLFK ERHLMELFGGKPMIYK + 2 OxPM SQHVQVEEGSEPDAFWEALGGK VSNGAGSMSVSLVADENPFAQGALR VSNGAGSMSVSLVADENPFAQGALR + OxPM IVPVDPATYGQFYGGDSYILVNYR MVRDPDQTDGPGLGYLSSHIANVER IEGDIKIVPVDPATYGQFYGGDSYILVNYR	29 1 1	HVVPNEVVVQR	1275.7111	Gsn Gsb	86287 5.83	3D-structure; Acetylation; Actin capping; Actin-binding; Alternative initiation; Calcium; Cytoplasm; Cytoskeleton; Direct protein sequencing; Phosphoprotein; Repeat; Secreted; Signal
X3_MOUSE	P48412	Glutathione peroxidase 3 [Precursor] EC 1.11.1.9 GSHPx-3 GPx-3 Plasma glutathione peroxidase GSHPx-P	35 4 19	FLVGPDGIPIMR FLVGPDGIPVMR + OxPM NSCPPTAELLGSPGR YVRPGGGFVPNFQLTEK	-	-	-	Gpx3	25589 8.26	Oxidoreductase; Peroxidase; Secreted; Selenium; Selenocysteine; Signal
13_MOUSE	P31898	H-2 class I histocompatibility antigen, Q10 alpha chain [Precursor]	170 13 40	FDSDAETPR GYLQYAYDGR GNEQSFMVSLR WEQAGAAEYYR TWTAADVAAITR KWEQAGAAEYYR AKGNEQSFMVSLR AYLEAECVEWLRR FIIVGYVDDTQFVR YFETSVSRPGLGEPR THVTHHPISDHEATLR APWMEQEGPEYWER APWMEQEGPEYWER + OxPM	64 2 9	FIIVGYVDDTQFVR YFETSVSRPGLGEPR	1671.9630 1694.9393	H2-Q10	37173 5.19	Glycoprotein; Immune response; Membrane; MHC I; Signal; Transmembrane
A_MOUSE	P01942	Hemoglobin subunit alpha Hemoglobin alpha chain Alpha-globin	-	-	138 2 21	IGGHGAEYGAEALER TYFPHFDVSHGSAQVK	1529.7271 1819.8743	Hba Hba-a1	15332 8.38	Direct protein sequencing; Heme; Iron; Metal-binding; Oxygen transport; Polymorphism; Transport
B1_MOUSE	P02088	Hemoglobin subunit beta-1 Hemoglobin beta-1 chain Beta-1-globin Hemoglobin beta-major chain	74 6 50	LLVVYPWTQR DFTPAAQAAFQK LLGNMIVIVLGHHLGK + OxPM VEAFNIDGLNHLDSLK KVITAFNDGLNHLDSLK YFDSFGDLSSASAIMGNAK + OxPM	28 1 6	LLVVYPWTQR	1274.7263	Hbb-b1	15813 7.26	3D-structure; Direct protein sequencing; Heme; Iron; Metal-binding; Oxygen transport; Phosphoprotein; Polymorphism; Transport
MO_MOUSE	Q91X72	Hemopexin [Precursor]	217 23 48	LWWLDLK GPDSVFLIK YWVYPPEK YYCTQGNK DVFVSCPGR WFWDFATR YWVYVYPEEK NPITSVDAAFR FNPVTGEVPPR KWFWDFATR WKNPITSVDAAFR GATYATTGSHYVVR CSPDPGLTALLSDHR GECQSEGVLFFQGNR SLPQPQKVNSLGLCSQ SGAQATWTEVSWPHEK GECQSEGVLFFQGNRK ELGSPPGELETDAAFSCPGSSR LFQEEFPGIPYPPDAAVECHR SLGPHTCSSNGSSLYFHGPNEYCYSSIDK	31 1 4	LFQEEFPGIPYPPDAAVECHR	2472.1731	Hpx Hpxn	52349 7.92	Glycoprotein; Heme; Iron; Metal-binding; Repeat; Secreted; Signal; Transport
4G1_MOUSE	P01868	Ig gamma-1 chain C region secreted form	80 8 46	APQVYTIPPPK VNSAAFPAPIEK SVSELPIMHQDWLNGK + OxPM DDPEVQFSWFVDDVEVHTAQTQPR TTPPQVYLALPSSAAQTNSMVTLGCLVK + OxPM SNWEAGNTFTCSVLHEGLHNHHTEK DCGCKPCICTVPEVSSVFIFPPKPK VTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPR	-	-	-	-	36252 7.23	3D-structure; Alternative splicing; Direct protein sequencing; Glycoprotein; Immunoglobulin C region; Immunoglobulin domain; Secreted
J_MOUSE	P01592	Ig J chain	56 5 27	AVFVTLPEIR CYTTMVPLR CYTTMVPLR + Oxidation (M) NPVVHLSEVCK EPSTEDPHEDIVER	-	-	-	Ig	18458 4.77	Glycoprotein; Signal
C_MOUSE	P01837	Ig kappa chain C region	63 5 74	SFNRNEC WKIDGSER NGVLNSWTDQESKDSTYSMSSTLTLTKDEYER + OxPM ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPK	-	-	-	-	11942 5.23	3D-structure; Direct protein sequencing; Immunoglobulin C region; Immunoglobulin domain
G1_MOUSE	O08677	Kininogen-1 [Precursor]	113 16 26	YVIEFIAR RPPGFSPFR SGNQVMLHR SGNQYMLHR + OxPM TDGSPTFYSFK CQALDMTEMAAR + OxPM ENEFFIVTQTCK ESNTELAEDCEK DAEEAATGECTATVGK RENEFFIVTQTCK FPSLHGDCVALPHGDDGECR HLGQSLDCNAIVYVMRPWENK EVLGHSIAQLIAENDHPFYYK HLGQSLDCNAIVYVMRPWENK + OxPM EFPSLHGDCVALPHGDDGECR AYFPCIGCVHASTDSPDLEPVLK	55 1 1	RPPGFSPFR	1363.5723	Kng1 Kng	74143 6.35	Alternative splicing; Blood coagulation; Glycoprotein; Inflammatory response; Protease inhibitor; Repeat; Secreted; Signal; Thiol protease inhibitor; Vasoactive; Vasodilator
3B_MOUSE	P01655	Ig kappa chain V-III region PC 7132	-	-	63 1 16	DIVLTQSPASLAVSLGQR	1855.3273	-	12357 4.6	Direct protein sequencing; Immunoglobulin domain; Immunoglobulin V region
SL_MOUSE	P01645	Ig kappa chain V-V region HP 93G7	63 5 50	LHSGVPSR LLIYYTSR DIQMTQTTSSLSASLGDR DIQMTQTTSSLSASLGDR + OxPM ASQDISNYLNWYQQKPDGTVK	47 1 7	LLIYYTSR	1328.5883	-	12317 7.97	3D-structure; Direct protein sequencing; Immunoglobulin domain; Immunoglobulin V region
6K_MOUSE	P34945	Ig kappa chain V-VI region NQ2-6.1	-	-	133 1 14	LLIYDTSNLASGVPVR	1717.9659	-	11823 8.98	Hybridoma; Immunoglobulin domain; Immunoglobulin V region
JC_MOUSE	P01872	Ig mu chain C region secreted form	127 14 33	DGPSVGPAPR NLVAMGCLAR NLVAMGCLAR + OxPM EFVCTVTHR KEFVCTVTHR YLATSQVLLSPK HPPAIVYLLPPAR GVASVCVEDWNHR GFSPADISVQWLQR LICEATHFTPKPFVSWLK VSTLTLTEEDWLNLHVYTCR DLHVPPAVAEMNPNVNVFVPPR DLHVPPAVAEMNPNVNVFVPPR + OxPM ESATVTCLVKGFSPADISVQWLQR	138 2 5	HPPAIVYLLPPAR GFSPADISVQWLQR	1330.7393 1633.7889	Igh-6	53625 6.56	Alternative splicing; Direct protein sequencing; Glycoprotein; Immunoglobulin C region; Immunoglobulin domain; Secreted

JG1_MOUSE	P28665	Murinoglobulin-1 [Precursor] MuG1	82 17 11	RPEEPPR VGAVTFSR HGPFFVK FR^VSMDK FQVENSNR FSTSQSLPASQTR DLFQCVSFIIRR DSSYSAFDDQNQER LQVTASPQSLCGLR AHFSYMSDILSSAIR AHFSYMSDILSSAIR + ORM EEHSFTVMEFVPR EEHSFTVMEFVPR + ORM VEFDLAFTPESWSLR LPFCFDYGMVPRSAPR LPFCFDYGMVPRSAPR + ORM MLRPLNELLPLAYIEDPK + ORM	-	-	-	Mug1 Mug-1	166463 6.38	Bait region; Direct protein sequencing; Glycoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal; Thioester bond
JP2_MOUSE	P11589	Major urinary protein 2 [Precursor] MUP 2	85 6 43	EKIEDNGNFR ENIDLSNANR LFLEQMVLEK FAKLCEEHGILR DGETFQLMGLYGR + ORM RVDEVAHFILAIDYR	-	-	-	Mup2	23935 5.34	3D-structure; Pheromone-binding; Secreted; Signal; Transport
JP8_MOUSE	P34938	Major urinary proteins 11 and 8 [Fragment] MUP11 and MUP8	128 13 72	IEDNGNFR EKIEDNGNFR ENIDLSNANR FAQLCEEHGILR DGETFQLMGLYGR DGETFQLMGLYGR + ORM RVDEVAHFILAIDYR TDYDNFLMAHLINEK + ORM LFLEQMVLEHSLVLK AGEYSVTYDGFNITTPPK	-	-	-	Mup8 Mup11	17723 4.85	Pheromone-binding; Secreted; Transport
YH14_MOUSE	Q6URW6	Myosin-14 Myosin heavy chain 14 Myosin heavy chain, non-muscle IIc Non-muscle myosin heavy chain IIc NMHC II-C	61 16 9	ASEWLMK GLEAEVLR GPLTFTR VTTEAEMK + ORM MMPPKFSK MMPPKFSK + ORM AAEQAASDLR EVVLQVDEER DEMAAEVASGNLSK QLLQANPLEAFGHAK ALEEEQEAREELER LAEFSQAAEEEEKVK HSQALVEMAEQLEQARR + ORM NTDQATMPDHTAAQKLCR LQRAQAELEVSTALSEAESK AAEQAASDLRTQVTELEDELTAAEDAK	-	-	-	Myh14	229359 5.49	Acetylation; Actin-binding; Alternative splicing; ATP-binding; Calmodulin-binding; Coil shape; Coiled coil; Motor protein; Myosin; Nucleotide-binding; Phosphoprotein
L13_MOUSE	Q8CCN1	NACHT, LRR and PYD domains-containing protein 13	63 7 15	LLLMATSSSQGR + ORM SRGAEEPAPGVR EHVRCLEER LSMVGAQEAVRVVSR + ORM ETVTSSCFTDKEQLK MVLVLQFSEEERTVFSSCFTDK SLLAMNLMELVDYLNQVCLNDYREIYR	-	-	-	Nalp13	77288 6.18	3D-structure; ATP-binding; Nucleotide-binding
IF_MOUSE	P97298	Pigment epithelium-derived factor [Precursor] PEDF Serpin-f1 Stromal cell-derived factor 3 SDF-3 Caspin	57 6 23	SSFVAPLEK TIQAVLTVPK SSFVAPLEKSVGTRPR TTLQDFHIDEDRYVR LTPLDYHLNQPFLFVLR SSASPTGHVLLSPLSVATALSALSLGAEHRTESIIHR	-	-	-	Serpinf1 Pedf Sdf3	46262 6.48	Direct protein sequencing; Glycoprotein; Secreted; Signal
VN_MOUSE	P23918	Plasminogen [Precursor] EC 3.4.21.7	282 31 51	FVDVWER LLEPNNR SSRPEFYK WCSEQTPHR NLEENYCR WEYCDIPR MRDVILFEK + ORM WEYCNLKR CEGETDFVCR HGITTPQTNPR YILGAHEEYLR CQSWAAMFPHR + ORM YCNIRVEYLNNIR GPWCYTTDPSVR GTVSVTYSGKTCQR DIALLKLSRPATITDK VIPACLPSPNYMVADR + ORM CTTPPPPSPTYQCLK TPENFPDAGLEMNYCR + ORM NPDGDVNGPWCYTTNPR NPDGEPRPWCFTTDPTK NPDGDKGPWCYTTDPEVR TICYTGNGETYQGTQAGR TAVTAAGTPCQGWAAQEPHR NPDGETAPWCYTTDSQLR VVSGCVAFPEVAPFVQSLR NPDGEPRPWCFTTDPTKR NPDNDEQGPWCYTTDPDKR YILQGVTSWGLGCARPNKPGVYVR FTGQHFCGGTLIAPEWVLTAAHCLEK WGAATPMVPNYSPSTHPFREGLEEHYCR	-	-	-	Plg	83463 6.21	Blood coagulation; Cleavage on pair of basic residues; Direct protein sequencing; Fibrinolysis; Hydrolase; Kringle; Protease; Repeat; Secreted; Serine protease; Signal; Tissue remodeling; Zymogen
CK_MOUSE	Q922Y4	Phosphoenolpyruvate carboxykinase, cytosolic [GTP] EC 4.1.1.32 PEPCK-C Phosphoenolpyruvate carboxylase	61 9 22	TNLAMMNPSLPGWK + ORM DGKPLWKPSPGEIVER EISFGSGYGGNSLLGKK VLEWMFGRIEGEDSAK + ORM YLAHWLSMLMKHRMLAKLPK IMTRMGISVLEALGDGEFIK + ORM GLGGYNVEELFGISKEFVEK YECYGDDAWVMKFDAQDHLR TNLAMMNPSLPGWK^ECY^GDDAWVMK + ORM	-	-	-	Pck1 Pepck	73351 6.18	Cytoplasm; Decarboxylase; Gluconeogenesis; GTP-binding; Lyase; Nucleotide-binding
BP_MOUSE	Q03734	Plasma retinol-binding protein [Precursor] PRBP RBP	-	-	166 2 13	YWGVASFLQR LQNLDGTCADSYSFVFSR	1226.6313 2379.9351	Rbp4	23533 5.69	Retinol-binding; Secreted; Signal; Transport; Vitamin A
A4_MOUSE	P31532	Serum amyloid A-4 protein [Precursor] Amyloid A-5 protein	91 6 43	YYTGIR YFQGLLNR KYFQGLLNR EAVQGTWDLWR RPHNFRPEGLPEKF DNLEANYQNADQYFYAR	-	-	-	Saa4 Saa5	15136 9.3	Acute phase; Amyloid; Direct protein sequencing; HDL; Secreted; Signal
A3K_MOUSE	P37759	Serine protease inhibitor A3K [Precursor] Serpin A3K Contrapsin SPI-2	131 11 31	FSIASNYR ELSELDER DLGLAEPHR TLPPSQIEELNLPK MQQVEASLQPETLR MQQVEASLQPETLR + ORM MQQVEASLQPETLRK NFRDEELSCSVLELK AVLDVAETGTEAAAATGVIGGIR ALYQTEAITADFQQPTEAK EFDPQDTFESEFYLDEKR	95 1 5	AVLDVAETGTEAAAATGVIGGIR	2142.2161	Serpina3k Mcm2 Spi2	47321 5.35	Direct protein sequencing; Glycoprotein; Protease inhibitor; Secreted; Serine protease inhibitor; Signal
TN_MOUSE	P43325	Tetranectin [Precursor] TN C-type lectin domain family 3 member B Plasminogen kringle 4-binding protein	60 5 21	QALQTVCLK EKQALQTVCLK AENCAALSGAAIVGK NRMDVLAQEVALLK AENCAALSGAAIVGKWFDK	-	-	-	Tna	22642 5.5	Lectin; Secreted; Signal
RB_MOUSE	P19221	Prothrombin [Precursor] EC 3.4.21.5 Coagulation factor II	168 16 29	VTGWGNLR QTVTSLLR LVENFCR VTVCDSVR ELLDSYIDGR YGFYTHVFR GIAPWQVMLFR GIAPWQVMLFR + ORM TTDAEHTFFNEK DNLSPRGQCETER SPQELLCGASLISDR REECSVPVCGQEGR KSPQELLCGASLISDR TFGLGEADCGLRPLFEK NPDSSTTGPWCYTTDPVVR ETWTTNIHEIQPSVLQVVNLPIVERPVCK	43 1 1	YGFYTHVFR	1189.5473	F2 Cf2	71649 6.34	3D-structure; Acute phase; Blood coagulation; Calcium; Cleavage on pair of basic residues; Gamma-carboxyglutamic acid; Glycoprotein; Hydrolase; Kringle; Protease; Repeat; Serine protease; Signal; Zymogen

41_MOUSE	P26039	Talin-1	-	-	29 1 1	LAQAAQSSVATITR	1416.5803	Tln1 Tln	271892 5.82	3D-structure; Cell membrane; Cell projection; Cytoplasm; Cytoskeleton; Membrane; Phosphoprotein; Structural protein
TE_MOUSE	Q92111	Serotransferrin [Precursor] Transferrin Siderophilin Beta-1-metal-binding globulin	221 29 43	DSAFQLLR SCHTGLQR VAQEHFGK SCHTGVDR IPSHAVVAR TVLPPDGPR WCALSHLER LLEACTFHKH EEYNGYTGAFR HTIFEVLPEK ASDTSITWNHLK LYLGHNYVTAIR SKDFQLFSSPLGK DQYELLCLDNTR SAGWVVPIGLLFCK QEDFELLCPDGTR YLGAEYMQGVGNMR TAGWNIPMGMLYNR YLGAEYMQSVGNMR + OxIM TAGWNIPMGMLYNR + OxIM KPVDQYEDCYLAR FDEFFSQGCAPGYEK ADRDQYELLCLDNTR NLKQEDFELLCPDGTR GDVAFVKNQTVLQNTEGK CAPNNKEEYNGYTGAFR DFASCHLAQAPNHVVVSR HQTVLDNTEGKNPAEWAK AVLTSQETLFGGSDCTGNFCLFK	94 2 4	LYLGHNYVTAIR DFASCHLAQAPNHVVVSR	1419.8383 2338.1463	Tf Trf	78841 6.94	Direct protein sequencing; Glycoprotein; Ion transport; Iron; Iron transport; Metal-binding; Methylation; Phosphoprotein; Repeat; Secreted; Signal; Transport
33_MOUSE	P155S3	Tripartite motif-containing protein 33 Down regulatory protein of interleukin-2 receptor	63 6 15	NLHSGIK LRLFCR NIIAVVAINK RTFQAPDLK AKICDEVQQDLQLQR GIQYRSYNTVPVSEYNLGVLGYPALSSGK	-	-	-	Trim33 Rpt-1 Rpt1	58376 6.56	3D-structure; Alternative splicing; DNA-binding; Metal-binding; Nucleus; Transcription; Transcription regulation; Zinc; Zinc-finger
4Y_MOUSE	P37369	Transthyretin [Precursor] Prealbumin	-	-	141 2 23	FVEGIYR TLGISPFHEFADVVFTANDSGHR	869.4333 2517.2361	Ttr	15883 5.77	3D-structure; Glycoprotein; Hormone; Retinol-binding; Secreted; Signal; Thyroid hormone; Transport; Vitamin A
28_MOUSE	Q9D1C8	Vacuolar protein sorting-associated protein 28 homolog Caspase-activated DNase inhibitor that interacts with ASK1 CIIA	59 7 49	LERAMDEIQPDLR LERAMDEIQPDLR + OxIM QVQGSEISSIDEFCRK YDNMAELFAVKTMQALEK + 2 OxIM AMDEIQPDLRELMETMSHR + 2 OxIM QTVSQWLQTLSGMSASDELDDSQYR FHGIPATPGVGAPGNKPELYEEVKLYK	-	-	-	Vps28	25721 5.37	Protein transport; Transport
DB_MOUSE	P21614	Vitamin D-binding protein [Precursor] DBP Group-specific component Gc-globulin VDB	132 16 46	SLSLLVSR VCSQYAAYGK HLSLLTTMSNR HLSLLTTMSRR + OxIM RTQVPEVFLSK KFSSSTFEQVNQLVK SCESDAPFPVHPGTPECCTK DLCQGSTTQAMDQVTFELSR DLCQGSTTQAMDQVTFELSR + OxIM VPTANLEEIVLPLAEDFTEILSR EVVSLTEECCAEGADPTCYDTR TLRECCDTQDVACFSTQSPLLK GFADQFLYEYSSNYQQAPLRLLVAYTK LCMAALSHQPQEFPTYVEPTNDEICEAFR KLCMAALSHQPQEFPTYVEPTNDEICEAFR + OxIM	118 1 4	VPTANLEEIVLPLAEDFTEILSR	2441.3169	Gc	55162 5.39	Actin-binding; Direct protein sequencing; Glycoprotein; Repeat; Secreted; Signal; Transport; Vitamin D
NC_MOUSE	P29788	Vitronectin [Precursor] Serum-spreading factor S-protein	-	-	139 2 7	GQYCYELDETAVRPGYPK LQDVWGIEGPIDAAFTR	2145.9903 2331.3453	Vtn	55613 5.67	Cell adhesion; Direct protein sequencing; Glycoprotein; Heparin-binding; Phosphoprotein; Repeat; Secreted; Signal; Sulfation
ZG_MOUSE	Q64726	Zinc-alpha-2-glycoprotein [Precursor] Zn-alpha-2-glycoprotein Zn-alpha-2-GP	63 6 24	LAFEPER CLAYGFYPDR AREEFLVTLK GFSQSLSTVDVDR YAYDGEDFIIFNK FQATAFLNDQAFFHYNSNSGK	-	-	-	Azgp1	35462 5.83	Glycoprotein; Pyrrolidone carboxylic acid; Secreted; Signal

Danksagungen

Mein außerordentlicher Dank gilt Herrn Prof. Dr. Jürgen Borlak für die exzellente wissenschaftliche Betreuung dieser Doktorarbeit und für die Möglichkeit diese am Fraunhofer ITEM anzufertigen. Ihre stete Diskussionsbereitschaft und unsere anregenden wie wertvollen Gespräche zu manch später Stunde werde ich in bester Erinnerung behalten.

Herrn Prof. Dr. Markus Kalesse danke ich für die freundliche Übernahme des Korreferates sowie des Prüfungsvorsitzes. Frau Prof. Dr. Kerstin Wydra sei für die Übernahme des Drittgutachtens gedankt.

Allen Kolleginnen und Kollegen der Abteilung Molekulare Medizin und Medizinische Biotechnologie am Fraunhofer ITEM danke ich für das angenehme Arbeitsklima der letzten Jahre. Hervorheben möchte ich Dr. Roman Halter für die Bereitstellung transgener Mäuse sowie meine aktuellen und ehemaligen Proteomics-Arbeitskollegen Dr. Sven Schuchardt, Ignazio Garaguso, Maria Stella Ritorto, Eckhard Belau, Giuseppe Gazzana und Antonello Calcutta für hilfreiche Diskussionen und technische Unterstützung. Dr. Susanne Reymann und Dr. Dörte Solle danke ich für ihre Hilfestellung in der Bioinformatik.

Meinen Eltern möchte ich für die Ermöglichung meines Studiums und für ihre moralische Unterstützung danken. Meiner gesamten Familie und meinen Freunden sei für ihre Unterstützung gedankt.

Ein besonderer Dank gebührt Milani Deb. Ich möchte Dir, Milu, für Deine Geduld mit mir, Deinen ungebrochenen Optimismus und Deine Zuneigung danken, ohne die ich bei weitem nicht dort stünde, wo ich heute bin.

Lebenslauf

Name	Bijon Gopal Chatterji
Geboren am	30. Dezember 1978 in Bremen
Eltern	Dipl.-Ing. Biswa-Gopal Chatterji und Tanuka Chatterji
Nationalität	deutsch
Anschrift	Adalbert-Stifter-Straße 9, 30655 Hannover
E-mail	bijon@chatterji.de

1985-1989	Grundschule in Bremen
1989-1991	Orientierungsstufe in Bremen
1991-1998	Gymnasium, Sekundarstufe 1+2 in Bremen
1998	Abitur
1998-1999	Zivildienst in Bremen
1999-2004	Studium der Biologie (Hauptfach: Biochemie/Biotechnologie, Nebenfächer: Molekulargenetik, Mikrobiologie), Diplomarbeit *„Fusionsproteine mit Leucinzippern als Dimerisierungsmodul"* bei Prof. Dr. Stefan Dübel am Institut für Biotechnologie und Biochemie der Technischen Universität Carolo-Wilhelmina in Braunschweig
2001-2003	Studentische Hilfskraft an den Instituten für Bioverfahrenstechnik und Biotechnologie/Biochemie, TU Braunschweig
2004	Diplom
2004	ZSB Studienpreis der TU Braunschweig
2004-2008	Doktorarbeit *„Serumproteomik zur Identifizierung tumorspezifischer Biomarker des nicht-kleinzelligen Bronchialkarzinoms"* bei Prof. Dr. Jürgen Borlak am Fraunhofer Institut für Toxikologie und Experimentelle Medizin in Hannover
2005-2009	Promotionsstudium der Biologie an der Gottfried Wilhelm Leibniz Universität Hannover
2009	Promotion zum Dr. rer. nat.
Seit 2008	Wissenschaftlicher Mitarbeiter im Bereich Serumproteomik und Proteinanalytik am Lehrstuhl für Pharmako- und Toxikogenomik der Medizinischen Hochschule Hannover

Veröffentlichungen

Chatterji B, Borlak J. Search for novel serum biomarkers of dysplasia in a transgenic mouse model of lung cancer. Submitted for publication **2009**.

Chatterji B, Borlak J. A 2-DE MALDI-TOF study to identify disease regulated serum proteins in lung cancer of c-myc transgenic mice. *Proteomics* **2009**, *9*, 1044-1056.

Chatterji B, Borlak J. Serum proteomics of lung adenocarcinomas induced by targeted overexpression of c-raf in alveolar epithelium identifies candidate biomarkers. *Proteomics* **2007**, *7*, 3980-3991.

Ritorto MS, **Chatterji B**, Borlak J. Neue Biomarker für Leberkrebs. *Biospektrum* **2007**, Sonderheft zur Biotechnica, 13. Jg., 14-15.

Chatterji B, Ritorto MS, Garaguso I, Belau E, Gazzana G, Borlak J. Onko-proteomics von Lungentumoren. *Biospektrum* **2006**, *7*, 732-735.

Tagungsbeiträge

Chatterji B, Borlak J. Serum proteomics of lung dysplasia. *18th International Mass Spectrometry Conference,* Bremen, 30 Aug-4 Sep **2009**.

Chatterji B, Borlak J. A proteomic study to identify disease regulated serum proteins in lung cancer of c-myc transgenic mice. *Proteomic Forum*, Berlin, 29 Mar-2 Apr **2009**.

Chatterji B, Borlak J. Identification of disease regulated serum proteins in lung cancer of c-myc and c-raf transgenic mice. *HUPO Annual World Congress*, Amsterdam, 16-20 Aug **2008**.

Chatterji B. Serum Proteomics of lung tumor bearing mice. *Mass Spectrometry Solutions for Life Science Research*, Hannover, 3 May **2007**.

Patent

Borlak J, **Chatterji B**. Serumproteomik zur Identifizierung tumorspezifischer Biomarker und für das therapeutische Monitoring in der Behandlung des nicht-kleinzelligen Bronchialkarzinoms. *Zum Patent angemeldet*