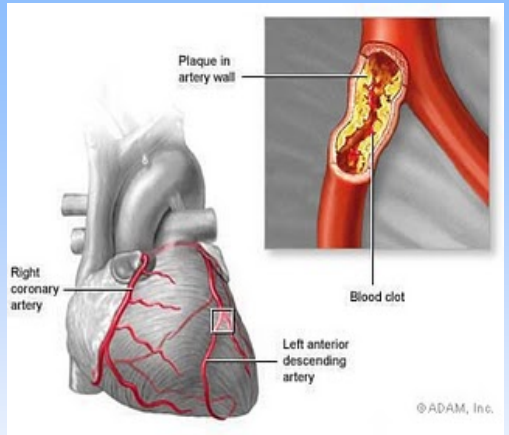
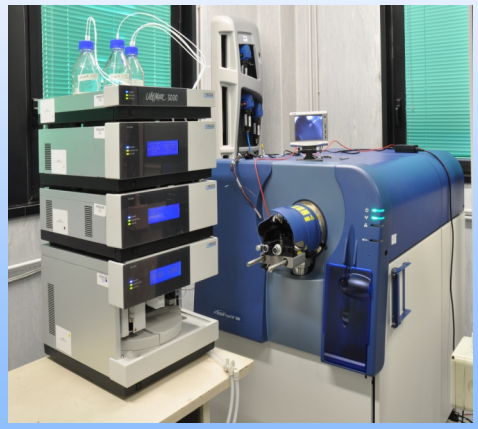


Proteomic Approaches in Cardiovascular Diseases

Antonella Cecchettini
Università di Pisa
Istituto di Fisiologia Clinica-CNR

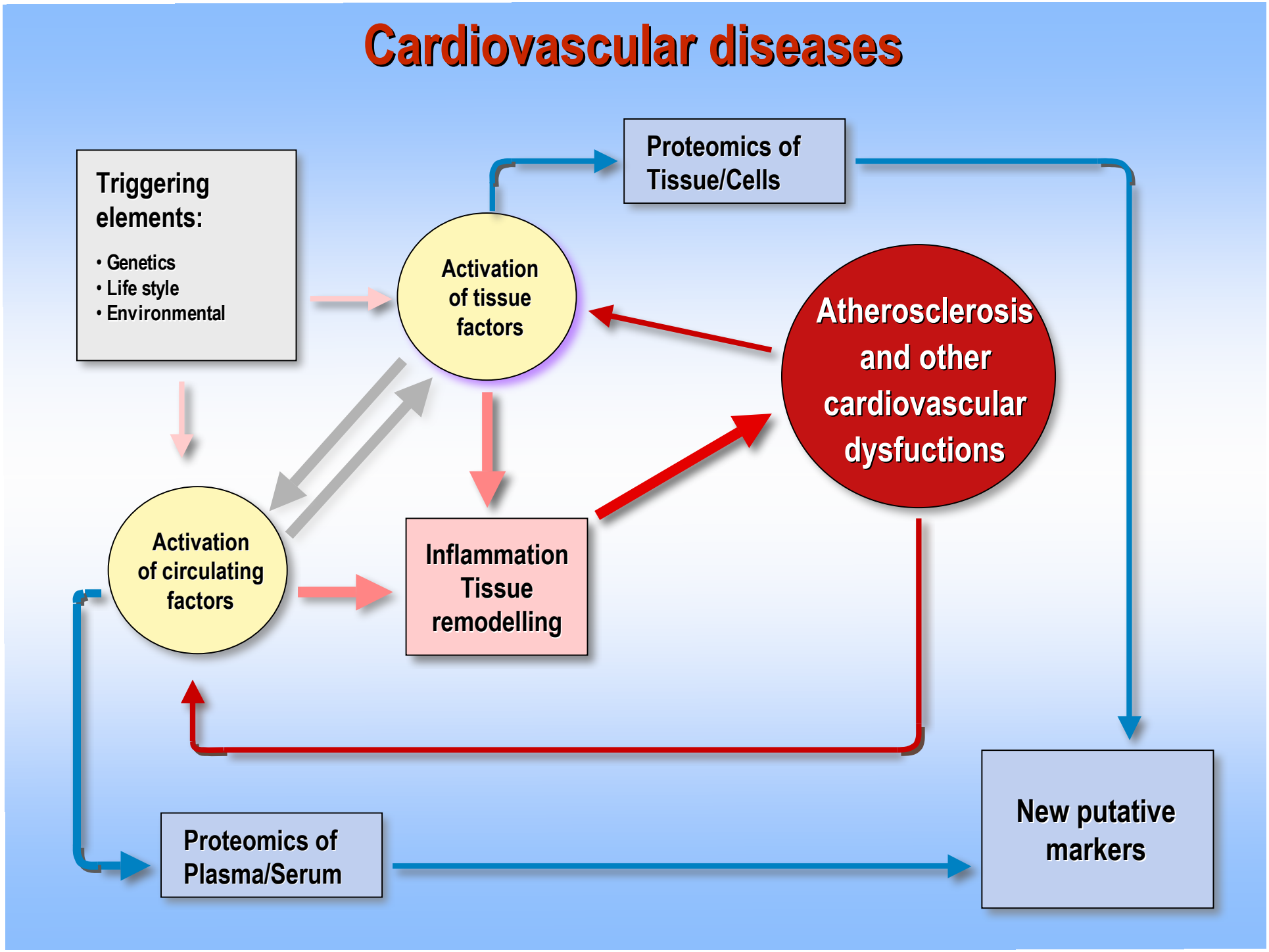


Proteomics is the science aiming at investigating and establishing the identity, quantity, structure and function of all proteins present in a tissue or in a cell, describing how these properties change with time, localization or physiological state.

Goals of Proteomics in Medicine

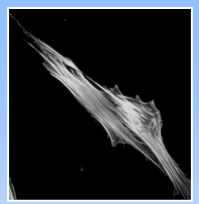
- Comparison between pathological and control samples to detect dis-regulated proteins
- Target identification for drug therapy
- Proteomics imaging of tissues
- Genomic and proteomics integrated strategy for system biology

BIOMARKER DISCOVERY


Cardiovascular diseases
Triggering elements:
• Genetics
• Life style
• Environmental
Proteomics of Tissue/Cells
Activation of tissue factors
Atherosclerosis and other cardiovascular dysfuctions
Activation of circulating factors
Inflammation Tissue remodelling
Proteomics of Plasma/Serum
New putative markers

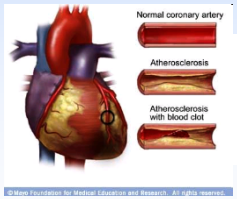
Primary Cells cultures

VSMCs



Experimental animals

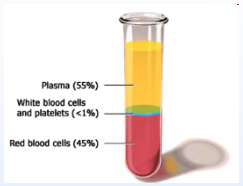
High Cholesterol Diet & Atherosclerosis



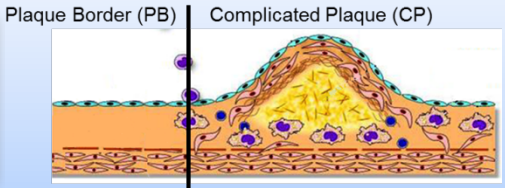
RTreat

Clinical investigations

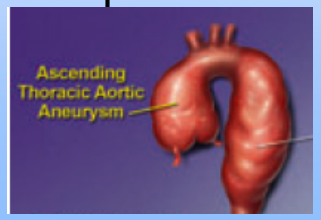
Plasma proteomics



Atherosclerosis: plaque secretome



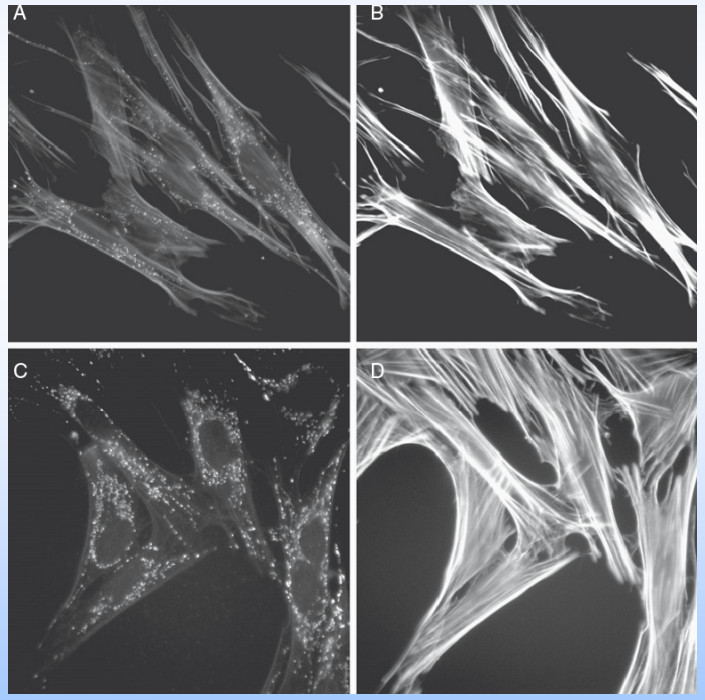
Hypertension and Aneurysms



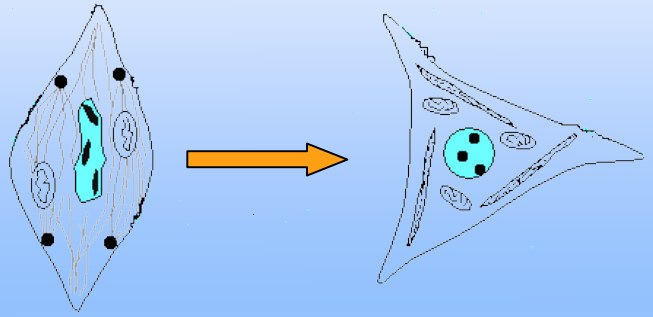
The cell model

For clinical investigations

For basic biological research



Adult VSMCs are highly specialized cells but retain also a great plasticity that allows them to undergo reversible phenotype changes (phenotype switch). Thanks to these characteristics they play a critical role in vascular repair. The other face of the coin of this high plasticity is that it prompts the cells to respond to environmental signals leading to the development of vascular diseases such as atherosclerosis, hypertension and vascular aneurysms.



The cell model

CHAPTER TWO

VASCULAR SMOOTH-MUSCLE-CELL ACTIVATION: PROTEOMICS POINT OF VIEW

Antonella Cecchettini,*,† Silvia Rocchiccioli,* Claudia Boccardi,* *and* Lorenzo Citti*

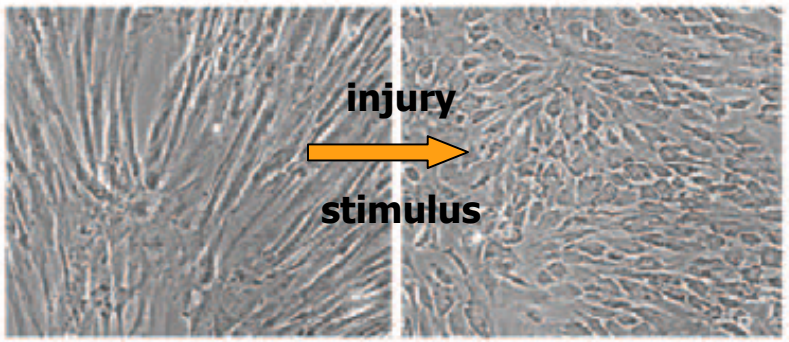
Tissue and Cell 38 (2006) 111–120

Resting smooth muscle cells as a model for studying vascular cell activation

Laura Poliseno[a], Antonella Cecchettini[b], Laura Mariani[a], Monica Evangelista[a], Fernanda Ricci[a], Franco Giorgi[b], Lorenzo Citti[a], Giuseppe Rainaldi[a,*]

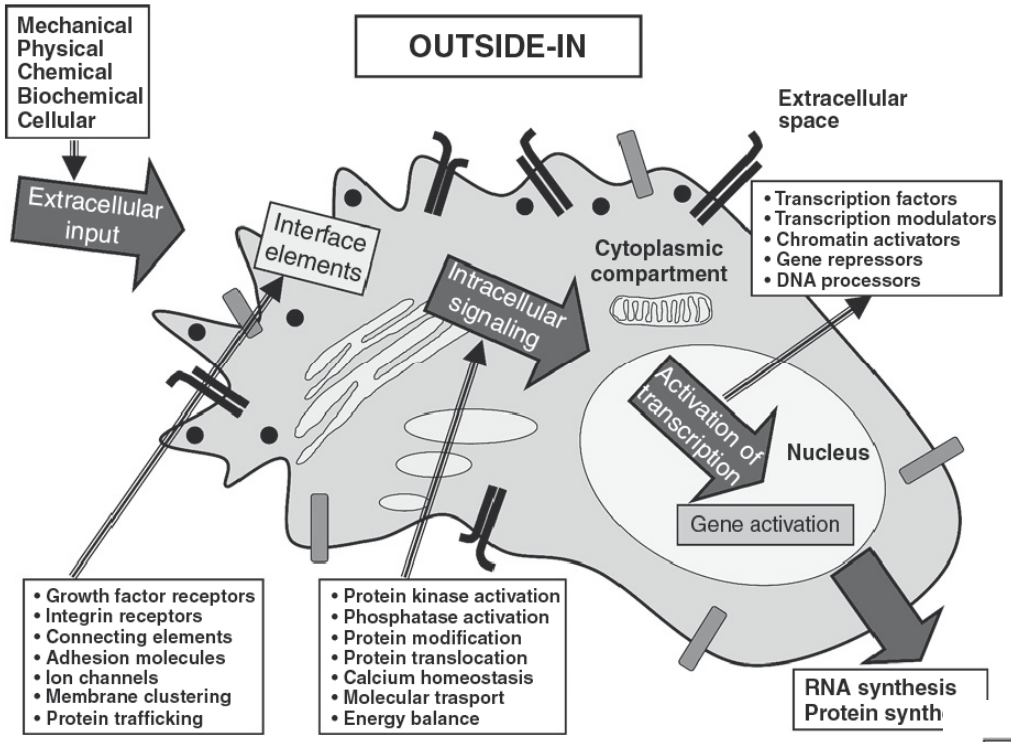
[a] *Laboratory of Molecular and Gene Therapy, Institute of Clinical Physiology, CNR, Area della Ricerca, Via Moruzzi 1, 56124 Pisa, Italy*
[b] *Department of Human Morphology and Applied Biology, University of Pisa, Italy*

Quiescent phenotype
- physiological conditions
- spindle-shaped morphology
- contractile activity
- low proliferation



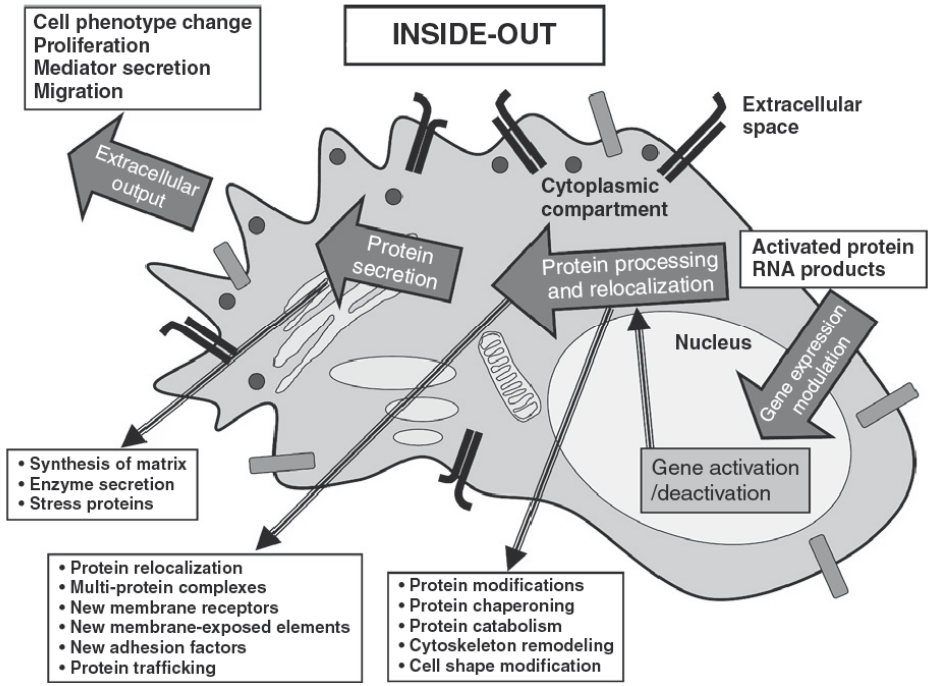
Activated phenotype
- pathological conditions
- romboid morphology
- synthetic-migratory activities
- high proliferation

VSMCs phenotype diversity is regulated by a wide range of elements including mechanical forces, endothelial-VSMC interactions and many growth factors.
A tight cross-talk between extracellular and cellular factors is responsible for coordinated "outside-in" and "inside-out" signals, generating conditions for the establishment of a given phenotype.

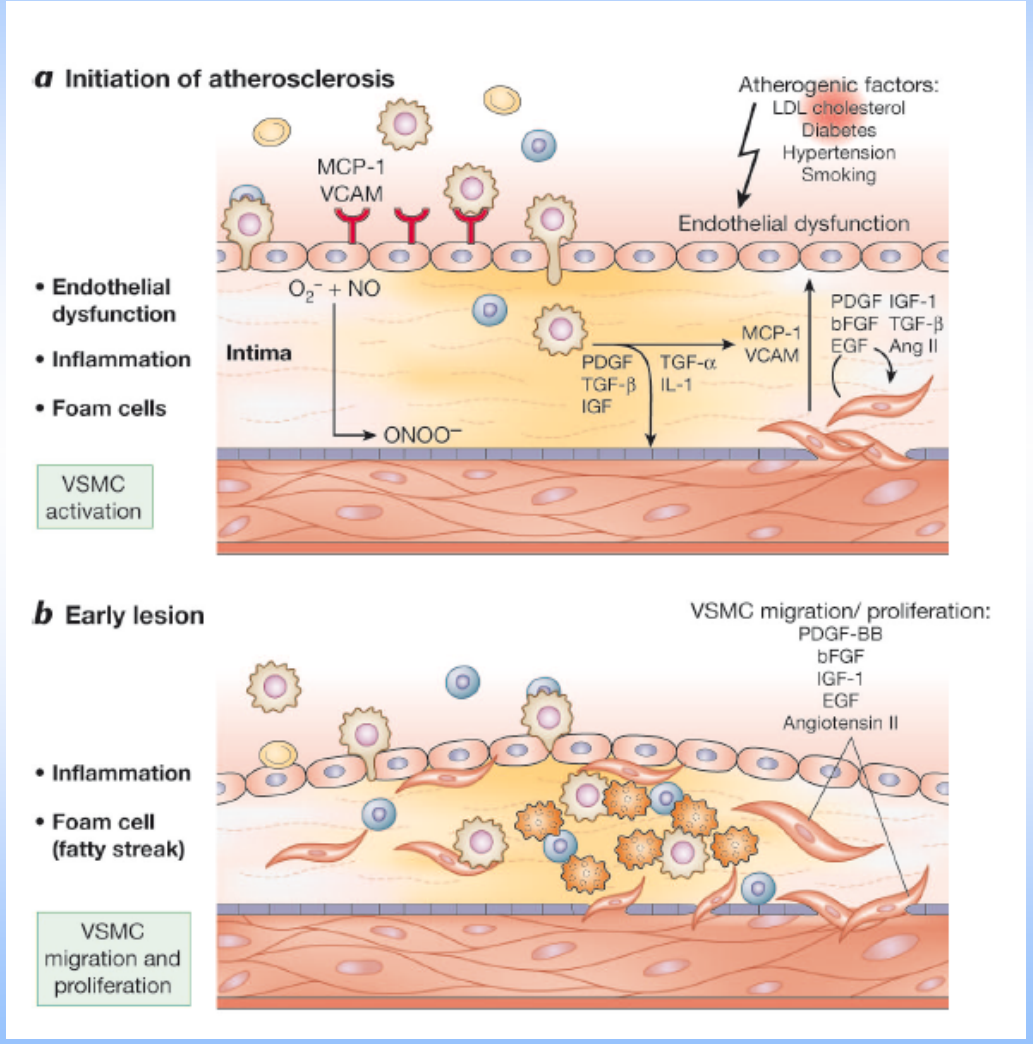


Cells sense the environment by means of dynamic surface elements whose response triggers signals that reach the nucleus where multiple genes are coordinately activated.

Modified and new synthesized proteins trigger dramatic changes (cytoskeleton remodeling, activation of new metabolic pathways, exposure of diverse repertoire of membrane proteins, secretions of new factors) that contribute to the establishment of a phenotype.



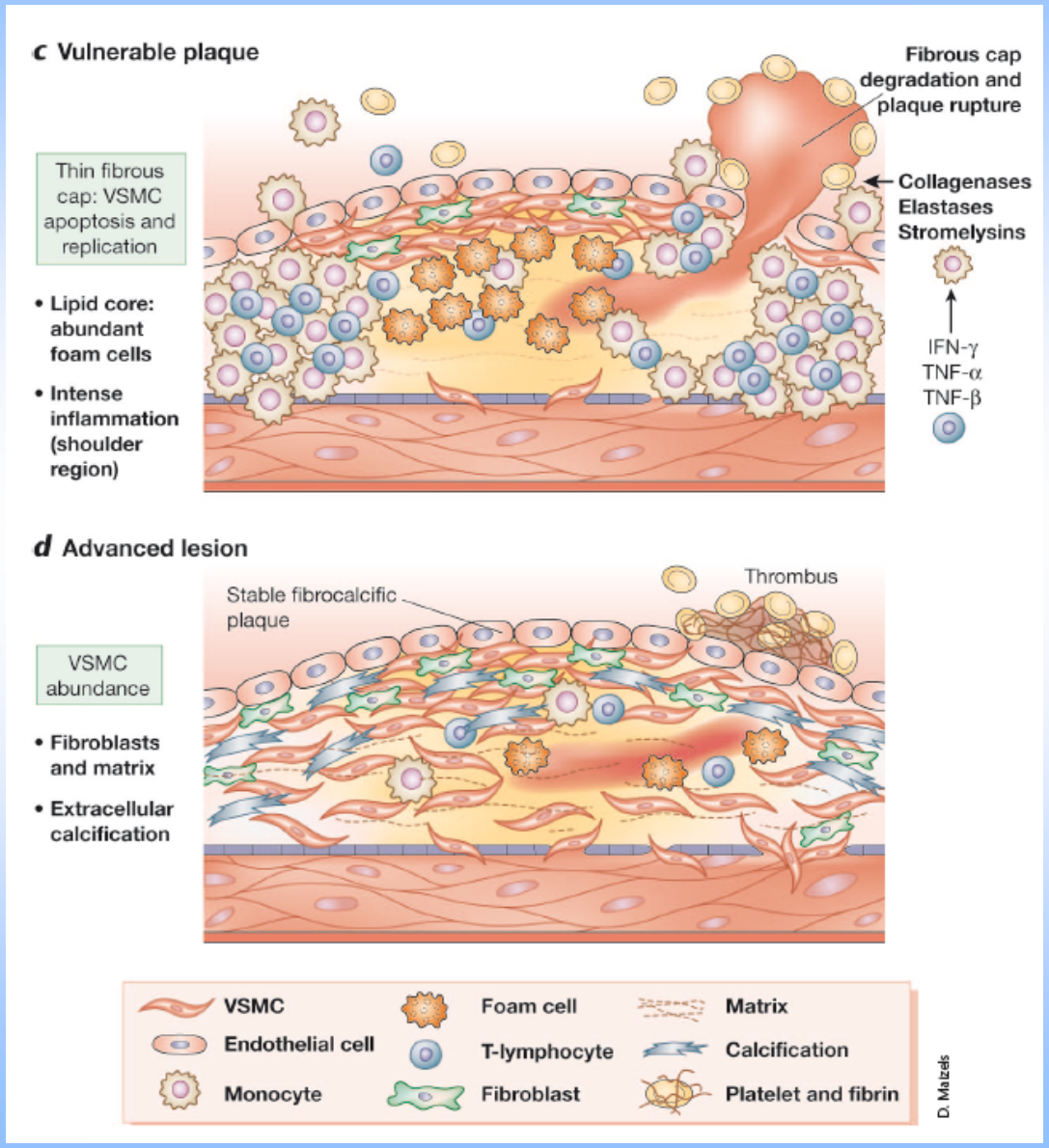
Role of VSMCs phenotype switch in Atherosclerosis development



NATURE MEDICINE • VOLUME 8 • NUMBER 11 • NOVEMBER 2002

Cardiovascular risk factors alter the vascular endothelium, which triggers a cascade of events, including the recruitment of leukocytes. Cytokines and growth factors are released by inflammatory cells and vascular cells.
VSMCs migrate, proliferate and synthesize extracellular matrix components on the luminal side of the vessel wall, forming the fibrous cap of the atherosclerotic lesion.

Role of VSMCs phenotype switch in Atherosclerosis progression



NATURE MEDICINE • VOLUME 8 • NUMBER 11 • NOVEMBER 2002

VSMCs may be important in maintaining the stability of the plaque through the formation of a stable fibrous cap.

Inflammatory mediators can induce thinning of the fibrous cap by expression of proteases, making the plaque weak and susceptible to rupture and thrombus formation.

In advanced disease, VSMCs are involved in extracellular calcification and give rise to fibrocalcific lesions.

Matter of fact → VSMCs are involved not only in lesion growth but also in the process of plaque rupture

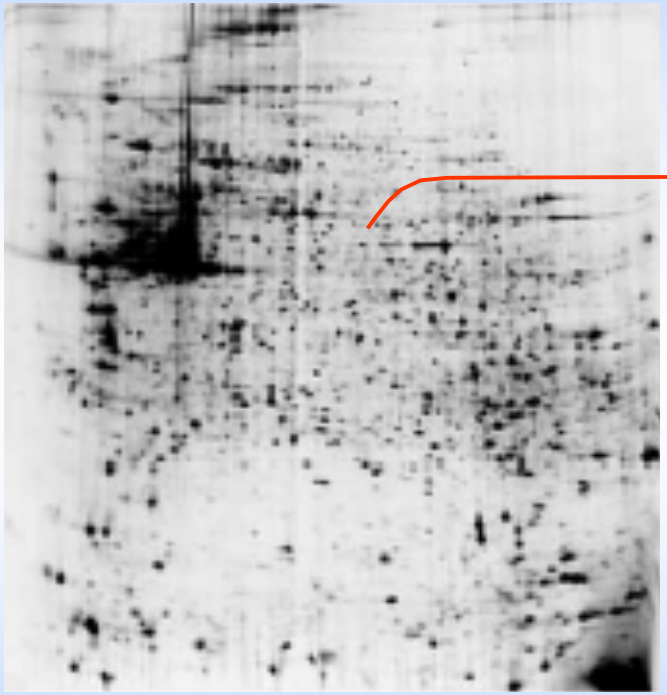
Unsettled questions →

(1) What are the key environmental signals that induce phenotypic modulation after vessel injury?
(2) What are the molecular mechanisms activated by these factors?
(3) What are the molecular biomarkers truly specific and unique for a given VSMC phenotype?
(4) Is it possible to hypothesize easy, quick, and non-invasive assays able to describe VSMCs state and thus to predict arterial wall condition?
(5) Is it possible to develop therapeutic strategies using molecular tools specifically designed against key elements in the activation pathways or eventually capable of reverting the phenotypic switching?

VSMC Proteome Mapping

.....in the beginning 2D-gel

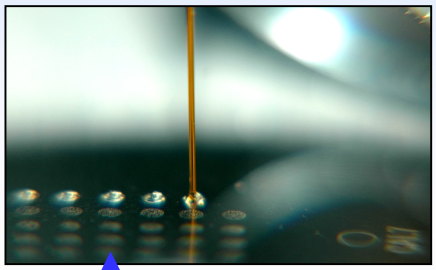
Image analysis of gels



Spot isolation

Trypsin digestion

Spotting on MALDI plate



MALDI TOF/TOF Mass-spectrometry of tryptic fragments

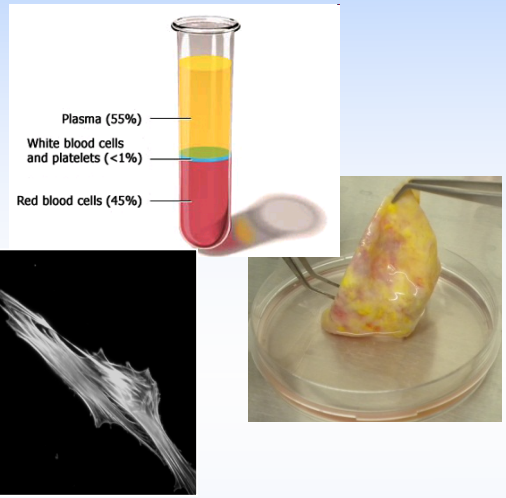
The first protein expression map of VSMCs was published in 2001 ***(McGregor et al. Proteomics 2001, 11:1405-1414)*****. The most detailed 2D-PAGE map was presented by Mayr et al who identified 235 proteins** ***(Proteomics 2005, 5:4546-4557)*** **.**

Challenges for 2-DE

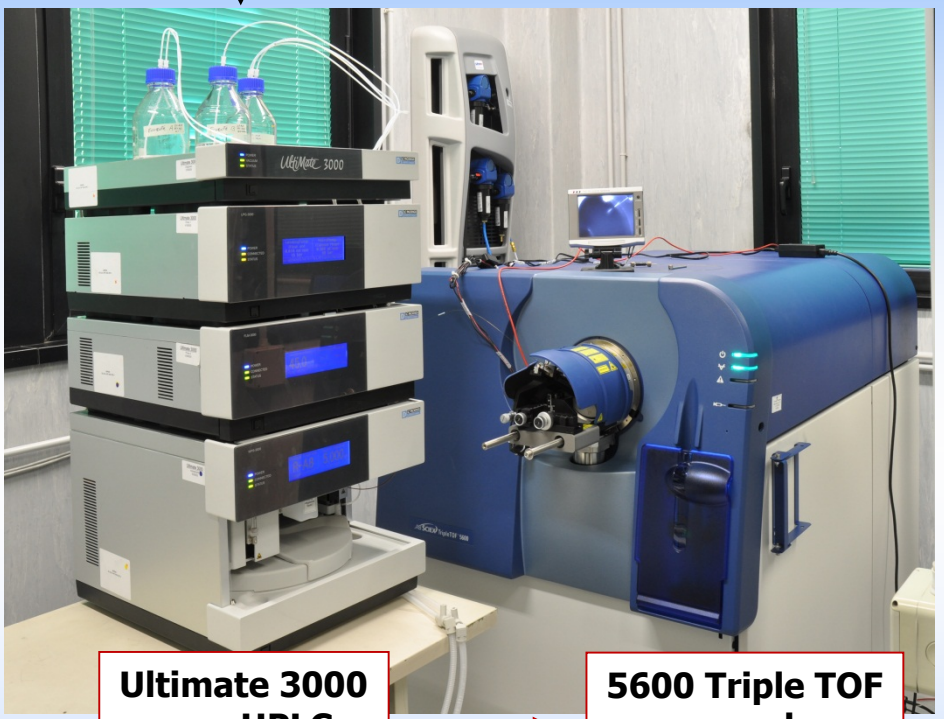
- Sensitivity
- Dynamic range of concentration must be adequate.
- It's impossible to display all proteins in one single gel.
- hydrophobic proteins
- Less expressed proteins are very difficult to detect, even employing most sensitive staining methods.
- Silver staining does not give reliable quantitative data.
- Reproducibility
- Time consuming

Gel-free PROTEOMICS

HPLC Fractioning & Mass analysis



Trypsin digestion

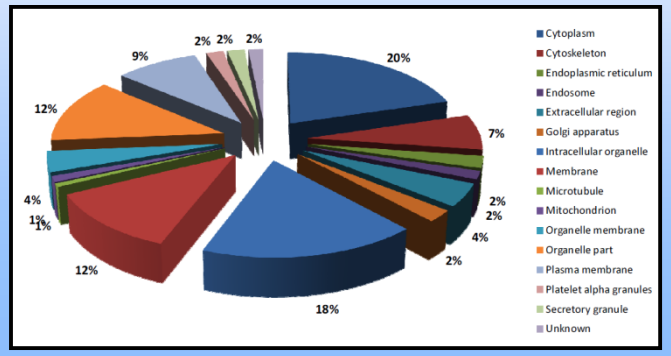


Ultimate 3000 nanoHPLC

5600 Triple TOF mass analyzer

in-line connection

Lysis, Centrifugation, Extract collection



Data evaluation & management

VSMC Proteome Mapping

.....coming across a gel free, sensitive and reproducible strategy.....

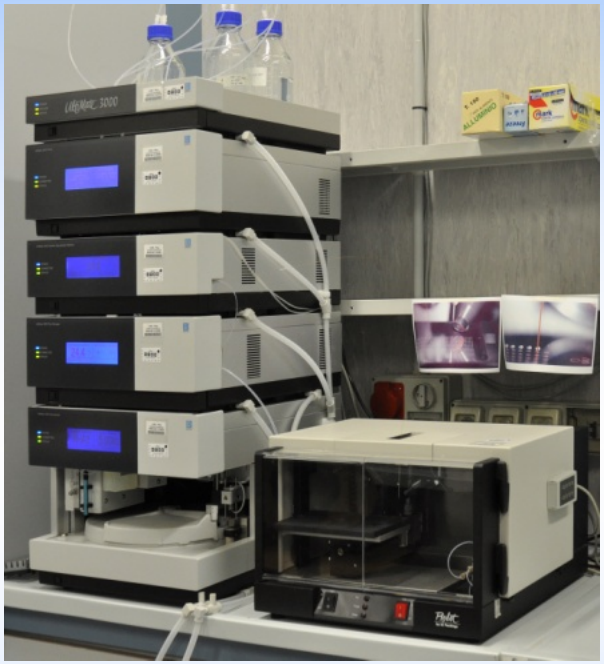
Rocchiccioli *et al. Proteome Science* 2010, **8**:15
http://www.proteomesci.com/content/8/1/15

PROTEOME SCIENCE

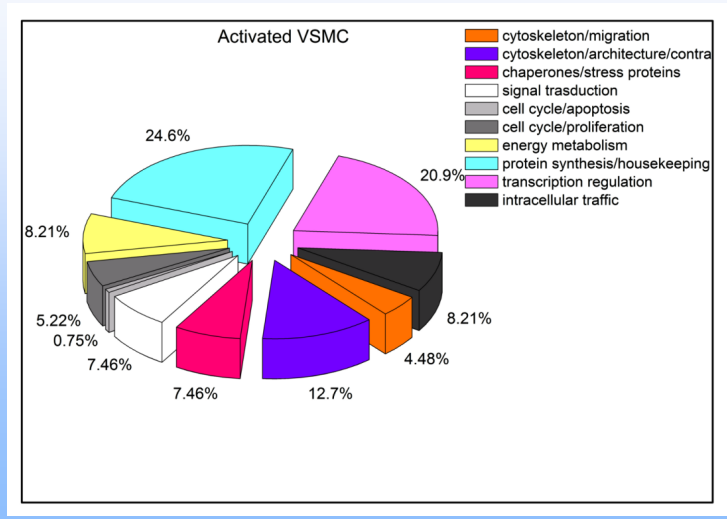
RESEARCH Open Access

A gel-free approach in vascular smooth muscle cell proteome: perspectives for a better insight into activation

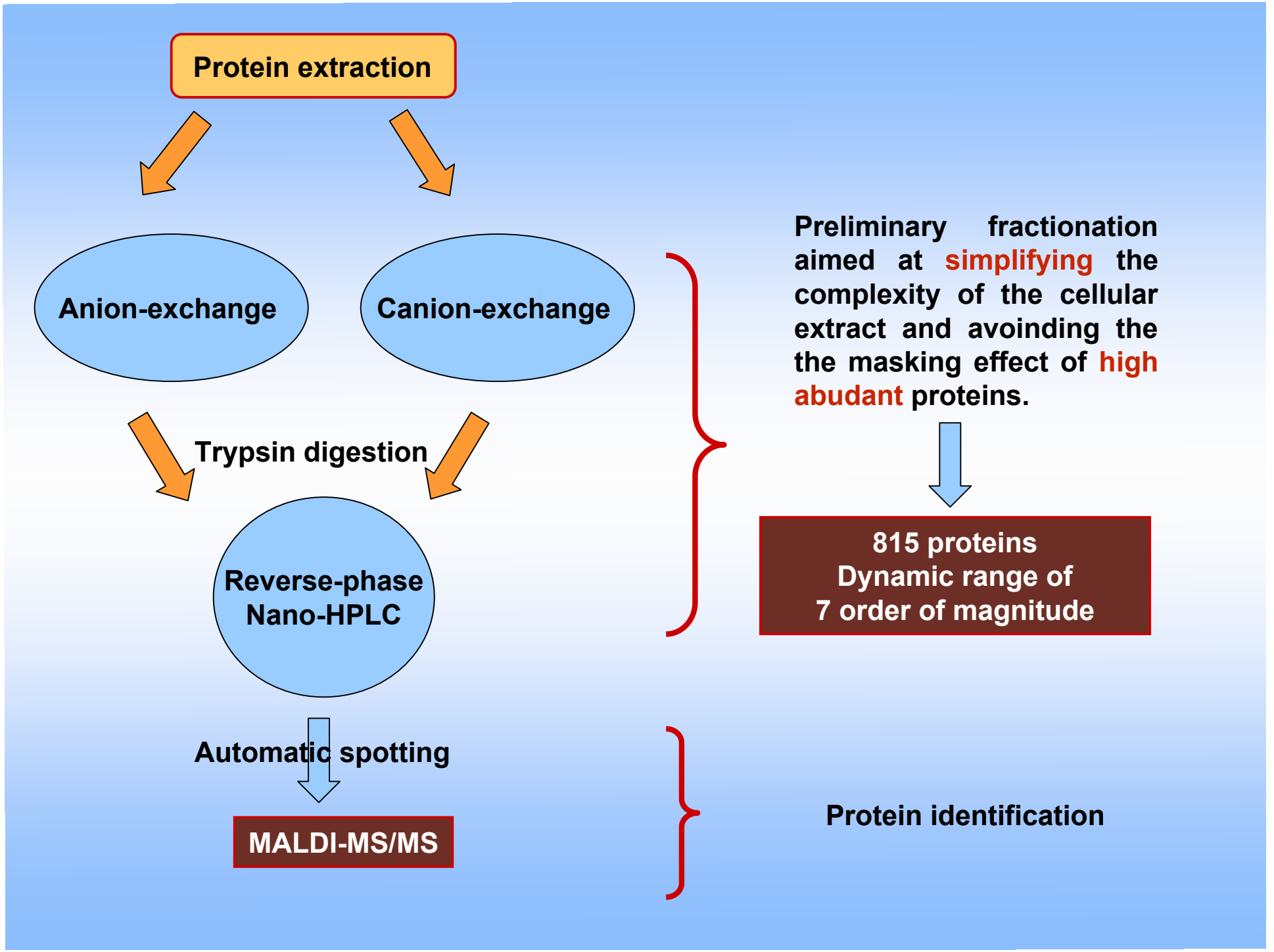
Silvia Rocchiccioli[1], Lorenzo Citti[1], Claudia Boccardi[1], Nadia Ucciferri[1,2], Lorena Tedeschi[1], Caterina Lande[1,2], Maria Giovanna Trivella[1], Antonella Cecchettini[2*]



815 proteins

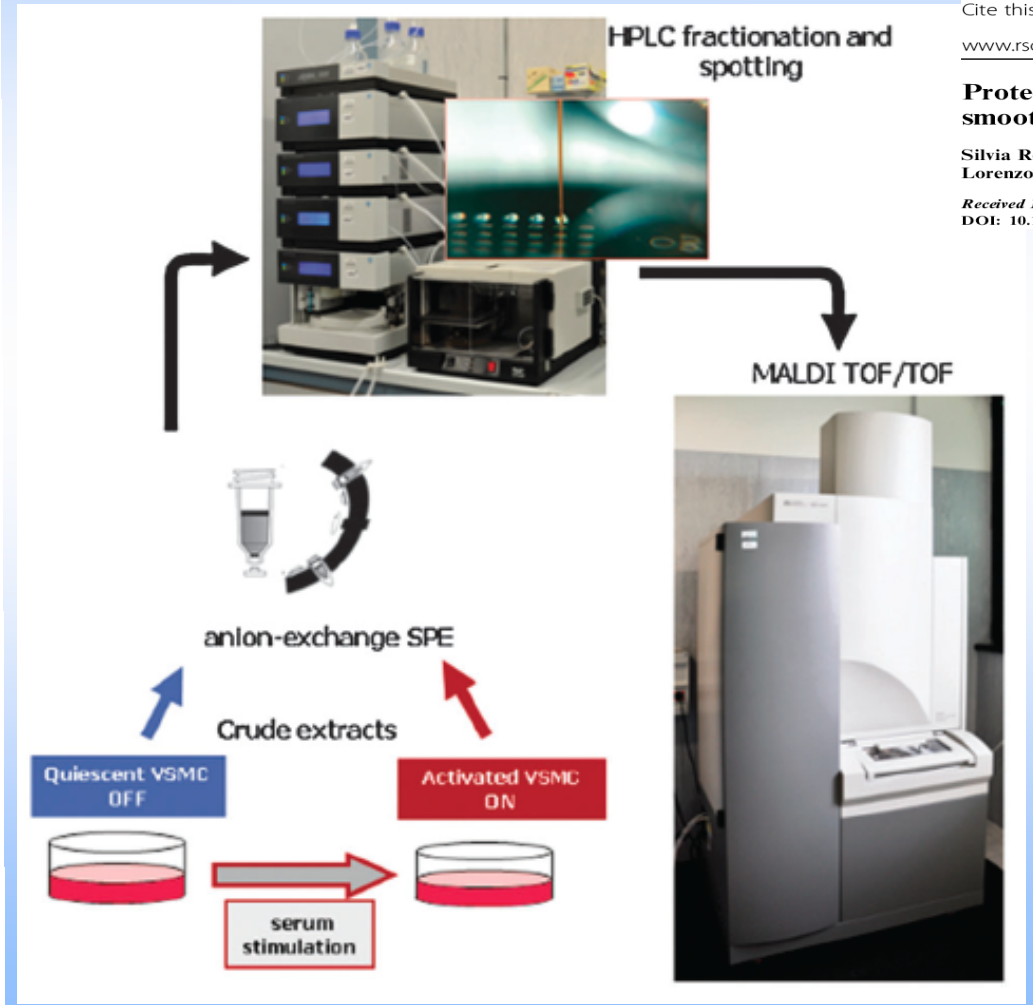


LCMALDI-TOF/TOF analysis combined with preliminary fractionation of a total protein extract is a powerful tool for biomarker discovery because of its high sensitivity and high throughput capacity.


Protein extraction
Anion-exchange
Canion-exchange
Trypsin digestion
Reverse-phase
Nano-HPLC
Automatic spotting
MALDI-MS/MS
Preliminary fractionation aimed at simplifying the complexity of the cellular extract and avoinding the the masking effect of high abudant proteins.
815 proteins
Dynamic range of
7 order of magnitude
Protein identification

Differential protein expression analysis: Insight in VSMC activation

.....availability of a suitable cell model and the assessment of a sensitive, reproducible workflow .....



Molecular BioSystems

Dynamic Article Links

Cite this: DOI: 10.1039/c2mb05470a

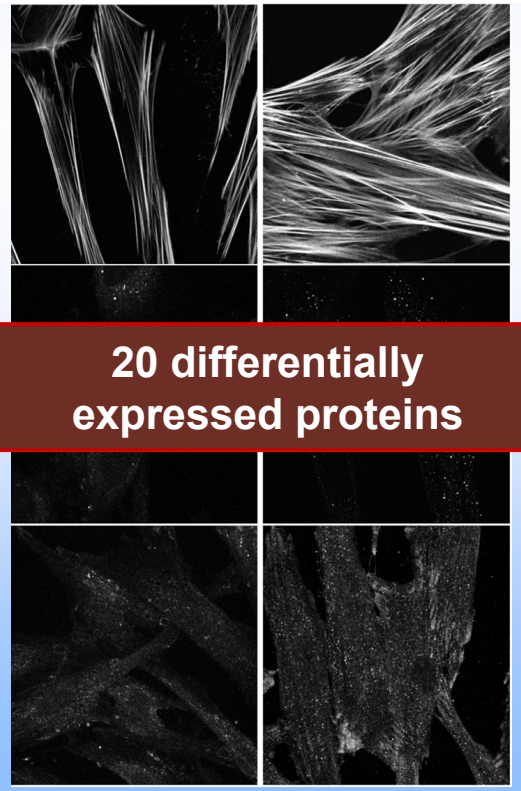
www.rsc.org/molecularbiosystems

PAPER

Proteomics changes in adhesion molecules: a driving force for vascular smooth muscle cell phenotypic switch†‡

Silvia Rocchiccioli,[a] Nadia Ucciferri,[ab] Laura Comelli,[a] Maria Giovanna Trivella,[a] Lorenzo Citti[a] and Antonella Cecchettini§[ab]

Received 14th November 2011, Accepted 2nd December 2011
DOI: 10.1039/c2mb05470a



20 differentially expressed proteins

.....allowed the visualization of a moving constellation.....

Table 1 Differentially modulated proteins in ON- *vs.* OFF-VSMCs

No.	Protein name	Accession number	Differential expression (ON/OFF)	Biological function	*p*-Value
1	Myosin 9	Q258K2	Up	Actin reorganization	4.2×10^{-4}
2	Myosin 10	P35580	Up	Actin filament movement, contraction	1.2×10^{-3}
3	Profilin-1	P07737	Up	Actin binding, migration	7.1×10^{-6}
4	PDI-A3	P30101	Up	Protein folding	1.1×10^{-3}
5	Fibronectin	P07589	Up	Cell adhesion and migration, integrins binding	5.7×10^{-6}
6	Calmodulin	P62157	Up	Calcium binding, contraction, proliferation	9.6×10^{-3}
7	Vimentin	P08670	Up	Cell shape maintenance, cytoskeleton stabilization	1.1×10^{-2}
8	Vinculin	P26234	Down	Focal adhesion structure and function regulator	1.0×10^{-2}
9	Transgelin	Q9TS87	Down	Actin binding, cytoskeleton modulator	2.1×10^{-3}
10	Transgelin 2	Q5E9F5	Down	Actin binding, cytoskeleton modulator	9.5×10^{-4}
11	Collagen alpha 1 (I)	P02453	Down	Extracellular matrix component	4.7×10^{-2}
12	Filamin A	P21333	Down	Actin- and integrin-binding	1.5×10^{-2}
13	LASP	Q99MZ8	Down	Actin-binding, cytoskeleton organization	1.5×10^{-3}
14	Talin-1	Q9Y490	Down	Plays a role in assembly of actin filaments, binds integrins and vinculin	2.9×10^{-2}
15	PDZ and LIM domain protein 1	Q5E9E1	Down	Adaptor molecules recruiting signaling molecules to the actin cytoskeleton	2.8×10^{-2}
16	Caldesmon	Q05682	Down	Calmodulin- and actin-binding, contraction regulator	1.5×10^{-2}
17	Thymosin β 4	P62328	Down	G-actin binding	1.3×10^{-2}
18	HSP 27	Q5S1U1	Down	Chaperone activity, inhibition of apoptosis	6.3×10^{-3}
19	Thioredoxin	P82460	Down	A major ROS-scavenging system	4.6×10^{-2}
20	Peroxiredoxin-1	Q5E947	Down	Antioxidant, regulator of signal transduction	1.9×10^{-6}

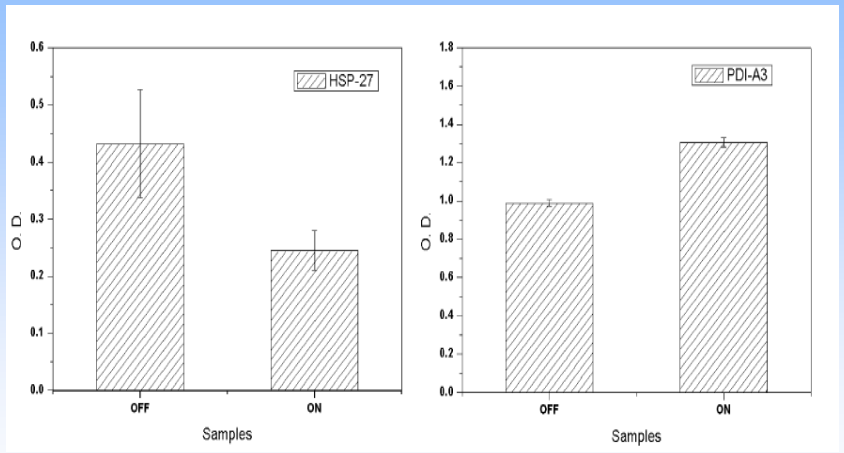
Variables were compared using Student's *t*-test and are reported as protein up- or down-regulation between ON- *vs.* OFF-cells.

- ✓Actin binding
- ✓Modulator of cytoskeleton
- ✓ integrin signaling.
- ✓Chaperones entailed in protein folding.
- ✓Cell contraction

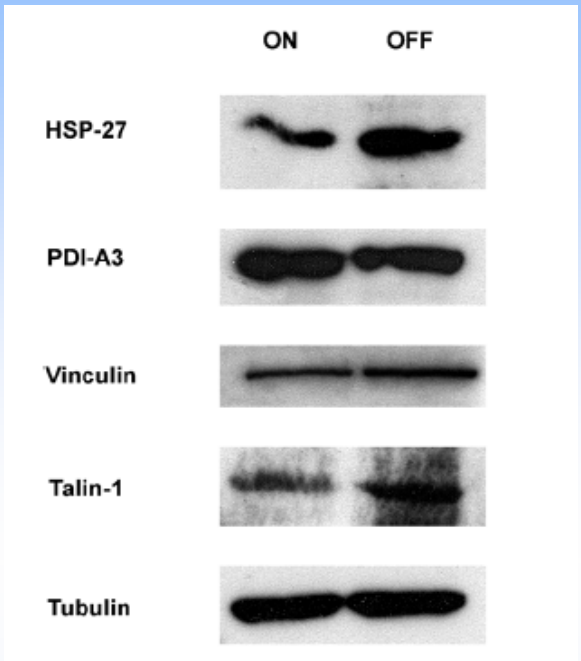
- **Vinculin**
- **Talin-1**
- **Fibronectin**
- **Vimentin**
- Filamin A
- LASP
- **PDZ and LIM domain protein 1**
- Caldesmon
- Thymosin b
- Profilin-1

→ **Cell migration**

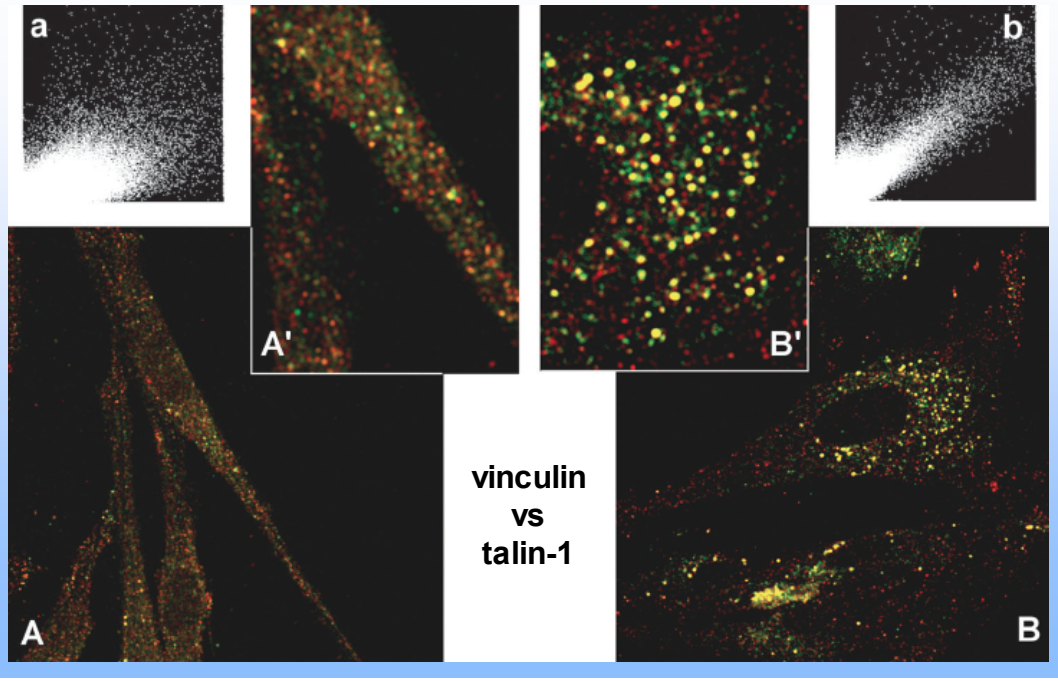
Elisa assay



Western blot



Confocal microscopy



★differentially expressed proteins are statistically confirmed

★and validated

★protein localization and co-localization change

Phosphoproteomics to disclose signaling networks in VSMC activation

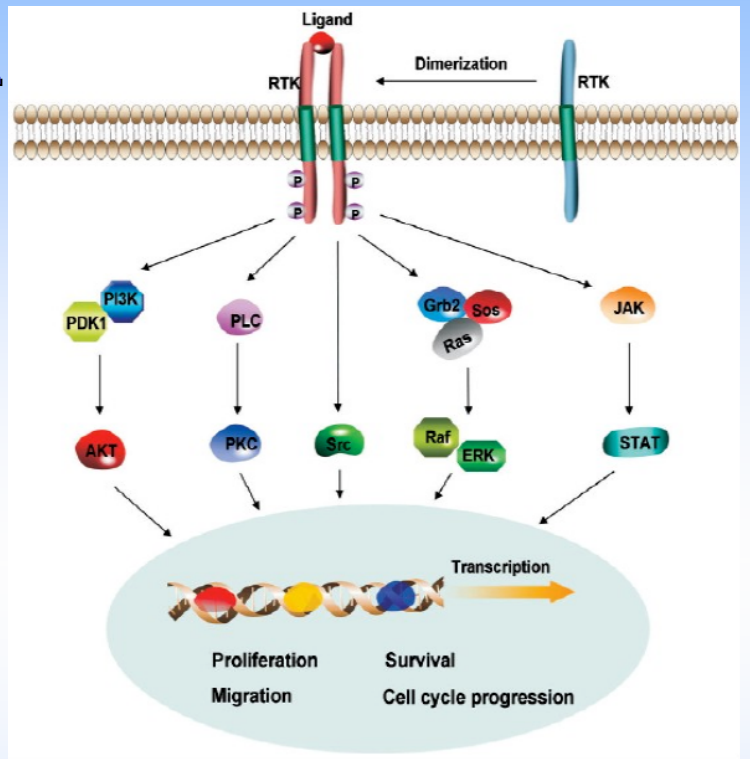
.....digging into the early phases of activation.....

Cell Tissue Res
DOI 10.1007/s00441-006-0357-3

REGULAR ARTICLE

A proteomic approach to the investigation of early events involved in vascular smooth muscle cell activation

Claudia Boccardi · Antonella Cecchettini · Anna Caselli · Guido Camici · Monica Evangelista · Alberto Mercatanti · Giuseppe Rainaldi · Lorenzo Citti



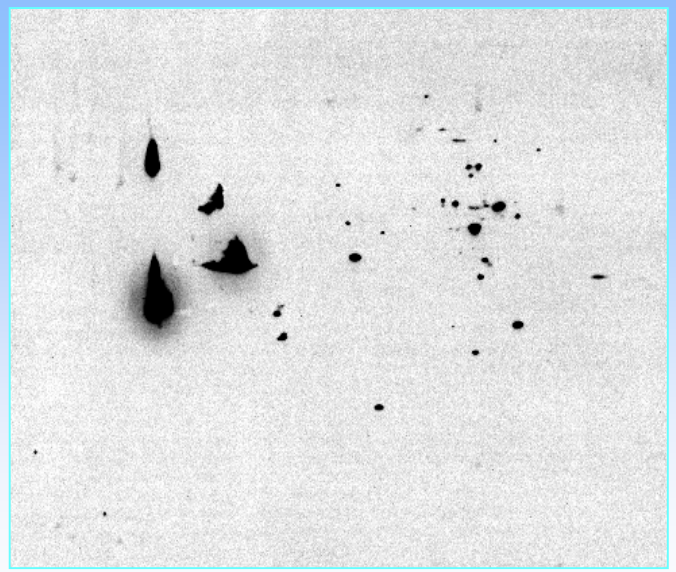
Growth factors play a major role in cell activation and among them PDGF has so far been the only one demonstrated to selectively promote phenotype switch.

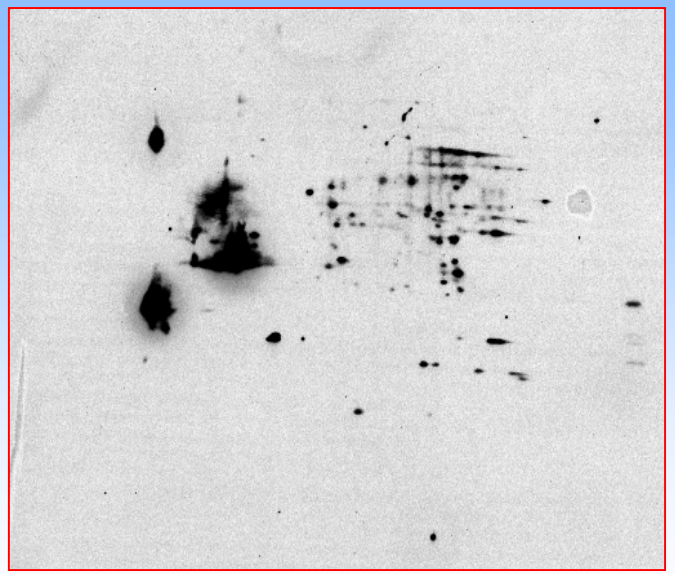
Phosphoproteome → because growth factors selectively and directly promote phenotype switch through the activation of signal transduction networks characterized by tyrosine kinases functions

Early stages → In order to identify:

★ triggering switches that could be suitable targets for therapeutic strategies

★ early markers of pathology for diagnosis

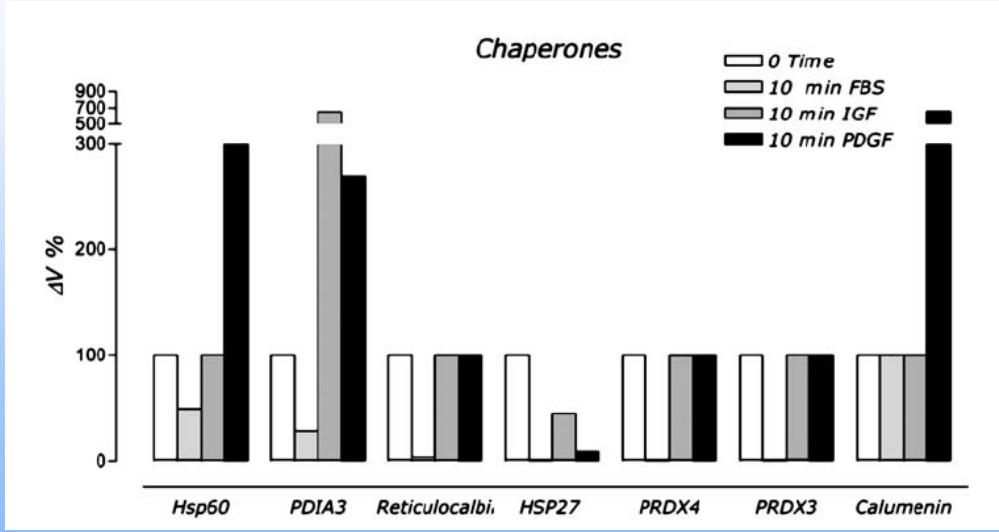




Quiescent

PDGF-activated

The most remarkable feature seems the recruitment of chaperones, i.e., molecules devoted to the make up and recycle of previously made proteins.



.....bumping into very precise and strong tools.....

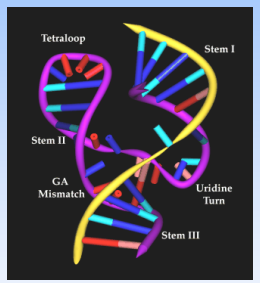
Drug Discovery Today • Volume 00, Number 00 • June 2009 REVIEWS

ELSEVIER

Hammerhead ribozymes in therapeutic target discovery and validation

Lorena Tedeschi[1], Caterina Lande[1], Antonella Cecchettini[2] and Lorenzo Citti[1]

[1] Institute of Clinical Physiology, National Research Council, CNR, via Moruzzi, 1, 56124 Pisa, Italy
[2] Department of Human Morphology and Applied Biology, University of Pisa, via Roma 55, 56126 Pisa, Italy



Hammerhead trans-acting ribozymes are the smallest RNA molecules capable of endonucleolytic activity that specifically bind and cleave RNA sequences of definite targets. Impressively, these precise molecular tools are able to discriminate between targets differing by a single nucleotide.

PDGF-r knock down effects VSMC activation

★ **Inhibits migration**

★ **Induces changes in chaperone phosphorylation**

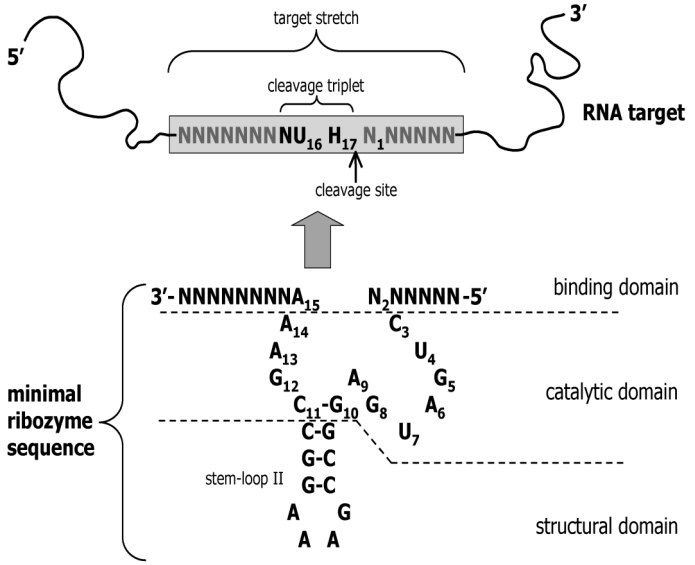
Lande *et al. BMC Research Notes* 2012, **5**:268
http://www.biomedcentral.com/1756-0500/5/268

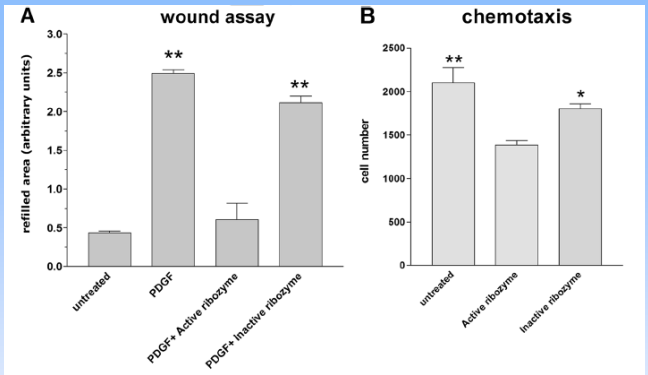
BMC Research Notes

SHORT REPORT Open Access

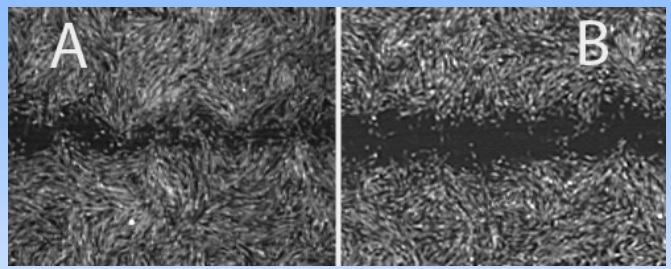
Ribozyme-mediated gene knock down strategy to dissect the consequences of PDGF stimulation in vascular smooth muscle cells

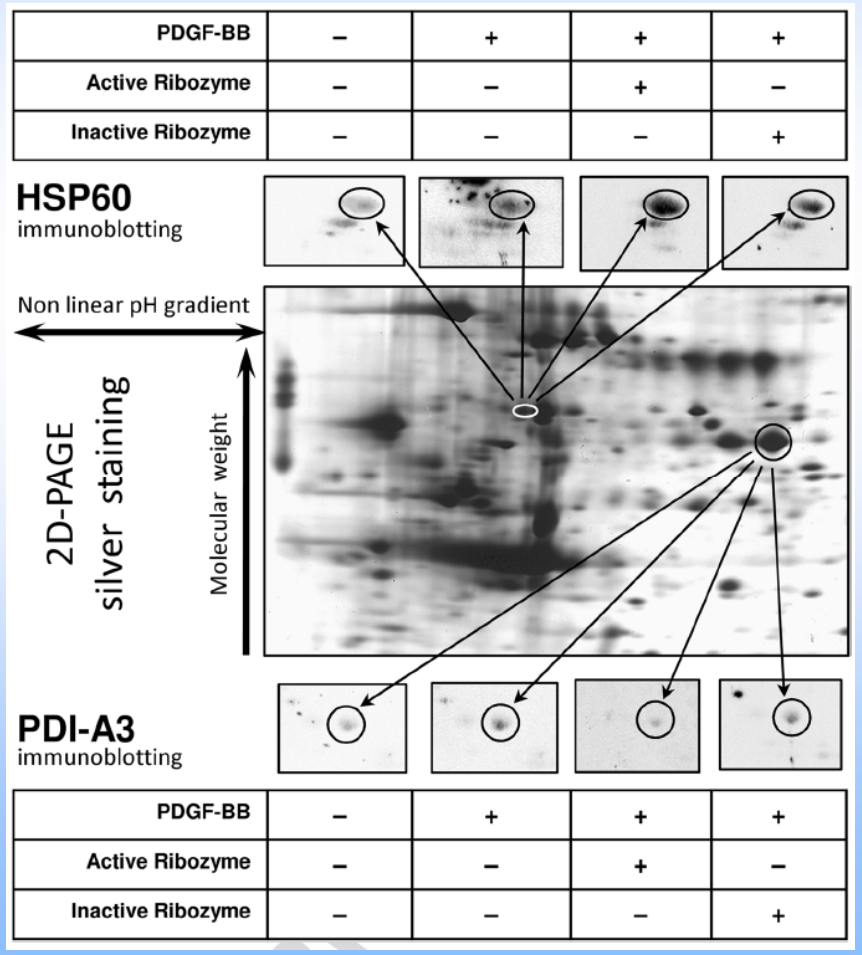
Caterina Lande[1,2,4*], Claudia Boccardi[3], Lorenzo Citti[1], Alberto Mercatanti[1], Milena Rizzo[1], Silvia Rocchiccioli[1], Lorena Tedeschi[1], Maria Giovanna Trivella[1] and Antonella Cecchettini[1,2]





Migratory activity is inhibited



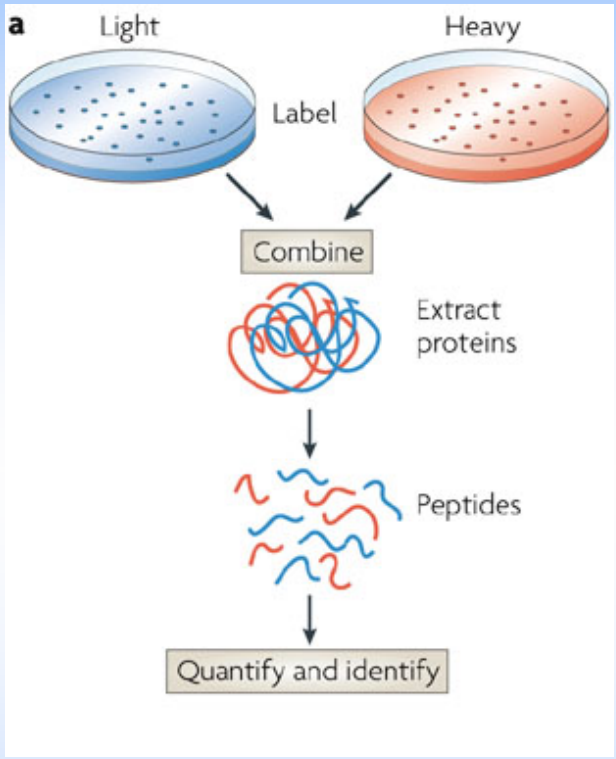


Phosphoylation profile of chaperones changes

Combined proteomics and gene knock down technologies enable the identification of key factors and key pathways involved in VSMC activation and eventually suggest crucial elements suitable for diagnostic evaluations and useful for planning new therapeutic strategies.

SILAC to evaluate time-course modifications in a quantitative manner

.....but the goal is quantitative phosphproteomics.....



Nature Reviews | Molecular Cell Biology

SILAC: metabolic incorporation of aa with stable isotopic nuclei

Strong Cation Exchange Chromatography: fractionation

SCX

IMAC

Immobilized-Metal Affinity Chromatography: Phosphpeptide enrichment

1300 phosphopeptides, clustering into 380 protein groups were identified. Among them, 21 proteins resulted phospho-modulated at different times (10', 2hr) upon PDGF-BB stimulation. These proteins are involved in cytoskeleton remodeling, focal adhesions, gap junction assembly and cell activation.