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The Fathers of Italian Histology

Scientific meeting
in memory of Carlo Rizzoli, Magister

Guest Editors

F.A. Manzoli, P. Carinci

under the auspices of
the University of Pavia, Italy

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The European Journal of Histochemistry was founded in 1954 by Maffo Vialli and published till 1979 under the title of Rivista di Istochimica Normale e Patologica , from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is published under the auspices of the Università of Pavia and of the Ferrata Storti Foundation, Pavia, Italy.
The European Journal of Histochemistry is the official organ of the Italian Society of Histochemistry and a member of the journal subcommittee of the International Federation of Societies for Histochemistry and Cytochemistry (IFSHC).

The Journal publishes original papers, technical reports, letters to the editor, review articles concerning investigations performed with the aid of biophysical, biochemical, molecular-biological, enzymatic, immunohistochemical, cytometric, and image analysis techniques.
Areas of particular interest to the European Journal of Histochemistry include:
- functional cell and tissue biology in animals and plants;
- cell differentiation and death;
- cell-cell interaction and molecular trafficking;
- biology of cell development and senescence;
- nerve and muscle cell biology;
- cellular basis of diseases

In memoriam of Carlo Rizzoli

Carlo Rizzoli was born on August 11, 1924 in Casalgrande, a small village near Reggio Emilia (Italy). On 1947 Carlo Rizzoli obtained his Medical Degree at the University of Bologna. He began his academic career at the Alma Mater under the directorship of Oliviero Mario Olivo, who headed the Chair of Histology and general embryology. He spent an intense period of study as a Research Assistant of Olivo, a direct descendant of Giuseppe Levi, a scientist of international renown and originator of the technique for growing embryonic tissues *in vitro*, mentor of three Nobel Laureates, Salvador Luria, Renato Dulbecco and Rita Levi-Montalcini. Olivo established a strong scientific collaboration with scientists of the Rockefeller Foundation in New York, were he spent a period of study under the guide of the Nobel Laureate Alexis Carrel, who afforded him appointments at the Rockefeller Foundation.

During this period, Carlo Rizzoli established the experimental approach for the study of the molecular basis of cell differentiation *in vitro*, anticipating some aspects of the present investigation on the potentiality of stem cells. Furthermore, Carlo Rizzoli was one of the first Italian scientists to publish its scientific reports in large-diffusion international journals, thus contributing to the world-wide diffusion of the seminal studies on the *in vitro* cell differentiation models.

In 1961, Carlo Rizzoli became Professor of Histology and general embryology and, since 1964 to 1999, Director of the Institute of Histology at the University of Bologna. The initial steps of this undertaking were challenging, since in 1963, following the recruitment of Oliviero Mario Olivo at the Chair of Human Anatomy, the facilities of the Institute of Histology were almost nonexistent. In few years, however, Carlo Rizzoli was able to organize an efficient research group of motivated young collaborators that included Paolo Carinci, Lia Guidotti, Francesco Antonio Manzoli, capable of introducing original and seminal lines of research into the national and international histological arena. In this way, a number of research programs has been undertaken, including the molecular studies on the

embryonic development, the modulating role of extracellular matrix macromolecules on gene expression, and the complex pattern of normal versus pathologic blood cell differentiation. With regard to this last issue, Carlo Rizzoli was the promoter of scientific collaborations between basic and clinical sides of the medical culture, strengthening a number of contacts with prominent Italian haematologists, contributing to the foundation of the Italian Experimental Haematology Group (GESI).

Carlo Rizzoli's scientific accomplishments led him to receive a number of recognitions and awards. Among them, he was Ordinary Fellow at the Academy of Sciences of the University of Bologna, he received the gold medal from the Ministry of the University and Research in 1979 and from the Ministry of Health in 1991. In the same year he awarded the Scanno Prize for medical research.

The prominence of Carlo Rizzoli in the scientific community is highlighted by an impressive amount of appointments. Since 1964 to 1972 it was Advisor in the Biology and Medicine Committee of the National Research Council, contributing to the release of the "Finalized proj-

ects'' to ensure an European dimension to the Italian research. Since 1968 to 1976 it was Dean of the Faculty of Medicine at the University of Bologna and, since 1976 to 1985, Chancellor of the University of Bologna. As Chancellor of the Alma Mater, Carlo Rizzoli had to face the most risky period of the student protest during the seventies; his mettle and cleverness succeeded in maintaining the balance between the authority of the institution and the requests of innovation. During this period he supported the development of research programs, the widening of the positions both of the teaching and technical staff, establishing a sound management at the University of Bologna.

Carlo Rizzoli was also appointed, since 1976 to 1989, as President of the CINECA, the most important institution for the electronic computation in Italy, endowing the Centre with the most powerful and up-to-date electronic computers available at that time. As President of the National Institute for Physical Training (ISEF), since 1965 to 1999, he founded the Seats of Verona and Catanzaro and obtained the recognition of the Physical Training Faculty into the Medical School. Carlo Rizzoli was among the founders and Member of the Board of Directors of the University ''G. D'Annunzio'' in Chieti, since 1976 to 1989, and it contributed to the development of the Medical School. As President of the Italian Society of Histochemistry, Carlo Rizzoli gave a strong contribution to the development of this branch of the morphological sciences.

The Italian histological school founded by Carlo Rizzoli includes a large group of his pupils and collaborators which head the Department of Histology or Human Anatomy in the Universities of Bologna, Ancona, Chieti, Ferrara, Genova, Perugia, Trieste, Parma, Urbino, Cassino.

Despite this impressive involvement in academic and administrative appointments, Carlo Rizzoli never neglected its role in teaching and mentoring. Thanks to the effort and the commitment of Carlo Rizzoli and Valerio Monesi, histology, which was an ancillary share of anatomy, rose to the dignity of a basic teaching. His Atlas of Histology, in cooperation with Carla Castaldini and Maria Antonietta Brunelli, and his contribution to the treatise of Histology formerly edited by Valerio Monesi are landmark textbooks which have been used by a generation of Italian students. Carlo Rizzoli was a fascinating speaker and left a strong and enduring mark on all of the pupils that have been the chance of listen his lectures. During the last period of his career, before its retirement, Carlo Rizzoli continued to teach with the same passion and involvement, joining at its scientific knowledge its wide experience and its foresight of the future development of the Medical Sciences.

In remembering Carlo Rizzoli, we celebrate his legacy his scientific flair, his impressive academic commitment, his wide classical culture. We will miss his many-sided personality, his skill in overcoming family tragic events by finding in the daily engagement the reasons of the existence.

Francesco Antonio Manzoli
Paolo Carinci

Introductory remarks

This supplement of the European Journal of Histochemistry is dedicated to the memory of Carlo Rizzoli.

The evaluation of the scientific contribution of Carlo Rizzoli to the evolution of the morphological sciences in Italy can be appreciated by considering the peculiar period of time, the fifties and the sixties of the past century, a crucial moment for the identification of the main fields of research which will characterize the impressive strengthening of cell biology. These trends were, from the beginning, based on either an analytical or a synthetic approach. The morphological trend, mainly based on the ultrastructural analysis of the fine cell organization into distinct components also analyzed by cell fractionation approaches, tended to dissect the cell organization and to analyze single events in an analytical way. A second trend, based on the tri-dimensional study of macromolecule organization, lead to the deciphering of the DNA structure, of the gene code and of the protein synthesis, integrating these topics into the analytic dissection of the cell. A third trend, which mainly utilized *in vitro* cell cultures and morpho-functional techniques, was aimed to consider the cell into its structural integrity in order to better describe its functions, mainly during the crucial events of embryonic development and tissue differentiation.

The evolution of the histological disciplines was mainly based on the first and third trend and in this area the scientific contribution of Carlo Rizzoli appears to be of fundamental impact. In fact, since its doctoral dissertation, dealing with the mechanisms of uptake of the yolk in the chick embryo, Carlo Rizzoli emphasized its interest towards the analysis of fundamental biological processes by means of biochemical and histochemical techniques. The brand of the scientific output of Carlo Rizzoli in this period was represented by the identification of the chemico-physical bases of tissue staining techniques, which were mainly based on empirical observations. In particular, the critical approach to histochemical techniques such as the Alcian and PAS staining, contributed to clarify the structural organization of the amorphous matrix of connective tissues, mainly of the cartilage. The wide use of *in vitro* cell culture methods also represented a key strategy, according to the lines of the Levi and Olivo school, that allowed Carlo Rizzoli to face the complexity of the cell functions in a olistic view, paving the way to the impressive evolution of the studies on the effects of regulatory factors on the differentiation of stem cells. On these bases, Carlo Rizzoli significantly contributed to the achievement of an innovatory discipline such as the histochemistry, not only by its scientific work, but also pursuing in introducing the discipline into the rules of the Medical School.

At the beginning of the seventies, the autonomy of the Histology with respect to other morphological disciplines, emerged owing to the wide knowledge about tissue differentiation mechanisms.

This situation required to be officially recognized, by including Histology into the fundamental curriculum of the Faculty of Medicine. Thanks to their academic ascendancy, Carlo Rizzoli, Valerio Monesi and Lorenzo Gotte, attained this recognition in 1975.

The increasing prominence of Carlo Rizzoli in promoting the policy of research as well as the wide involvement in academic appointments, as Dean of the Faculty of Medicine and Chancellor of the University of Bologna, and in national agencies of the research and public health, including the National Research Council and the Health Superior Council, partly demanded its attention and involvement, so that the continuity of the School was pursued by Paolo Carinci and Francesco Antonio Manzoli. The group of Carinci has been mainly involved in studies concerning the mechanism of control of the synthesis of the extracellular matrix and on its role in modulating the embryonic development, and the Manzoli's group in the identification of the functional role in cell proliferation and differentiation of a signalling system based on inositol lipids located at specific nuclear domains.

The many-sided scientific personality of Carlo Rizzoli was based on an unusual ability in maintaining a wide cultural open-mindedness (from the statistics to the organic chemistry) and the

stringency in applying this knowledge to specific research aims. Its unique personality contributed not only to the admiration but also to the fascination and affection of his pupils and followers.

On April 21, 2007, a Symposium, dedicated to memory of Carlo Rizzoli, has been held at the Institute of Human Anatomy of the University of Bologna. The contributions of the participants to the Symposium represent a sort of *florilegium* of the main results obtained in the last years by the large group of pupils, friends and colleagues of Carlo Rizzoli, which, in this way, want to witness their belonging to a common cultural adventure.

Paolo Carinci
Francesco A. Manzoli

The Fathers of Italian Histology
Scientific meeting in memory of Carlo Rizzoli, Magister

Bologna, April 21st, 2007
Aula Olivo - Dipartimento di Scienze Anatomiche Umane
University of Bologna

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Osteogenic and chondrogenic differentiation: comparison of human and rat bone marrow mesenchymal stem cells cultured into polymeric scaffolds

B. Zavan,[1] C. Giorgi,[1] G.P. Bagnara,[2] V. Vindigni,[1] G. Abatangelo,[1] R. Cortivo[1]

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Hyaluronan-based scaffold were used for *in vitro* commitment of human and rat bone marrow mesenchymal stem cells (MSC). Cells were cultured either in monolayer and in 3D conditions up to 35 days. In order to monitor the differentiating processes molecular biology and morphological studies were performed at different time points. All the reported data supported the evidence that both human and rat MSC grown onto hyaluronan-derived three-dimensional scaffold were able to acquire a unique phenotype of chondrocytes and osteocytes depending on the presence of specific differentiation inducing factors added into the culture medium without significative differences in term of time expression of extracellular matrix proteins.

Key words: mesenchymal stem cell, bone, cartilage, hyaluronan

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Stem cells, essential building blocks of multicellular organisms, are capable of both self-renewal and differentiation into at least one mature cell type. Stem cells are extremely versatile, differentiating as a function of when and where they are produced during development. The best characterized are embryonic stem cells (ESCs) derived from very early embryos. These cells proliferate indefinitely in culture, while retaining the capacity to differentiate into virtually any cell type when the appropriate site of the developing organism is reached. Thus, ESCs can generate large quantities of any desired cell useful for clinical purposes (Jorgensen C, *et al.* 2004). Stem cells collected from adult tissues or older embryos appear more restricted in their developmental potential, their ability to proliferate, and their capacity for self-renewal. Human bone marrow has a multipotent population of cells capable of differentiating into a number of mesodermal lineages. Mesenchymal stem cells (MSCs) are, in fact, the progenitors of all connective tissue cells. MSCs have been successfully isolated from the bone marrow of a variety of species including human, rat; dog; mouse and rabbit (Radice *et al.* 2000). After expansion in culture, they differentiate into several tissues such as bone, cartilage, fat, muscle, tendon, liver, kidney, heart, and even brain cells (Alhadlaq A *et al.* 2004). Due to their multilineage differentiating potential, and to their capacity to undergo extensive replication without losing this capacity, MSCs have enormous potential in the fields of cell therapy and tissue engineering. These cells can be induced to differentiate when submitted to specific environmental factors; however, to regenerate a true functional human tissue for *in vivo* application, it is necessary the use of fully characterized MSC and scaffolds. The behaviour of MSC embedded in biomaterials, in the long term and in the context of pathological joints,

remains to be studied before clinical application can take place. On the light of these considerations in the present study, we compared the differentiation of MSCs collected from two of the most utilized bone marrow species: human and rat.
Using tissue engineering techniques and hyaluronan (HA) derived biopolymers as supporting scaffolds for three dimensional *in vitro* cell culture, MSCs were stimulated to give rise to bone and cartilage tissue. Biopolymers (HYAFF^{tm} biomaterial, Fidia Advanced Biopolimers, Abano Terme, Padova, Italy) have been extensively studied for *in vitro* reconstruction of tissues such as epidermis, dermis and cartilage (Tonello C, *et al.* 2005; Brun *et al.* 1999). These engineered tissues are used in clinical practice for the treatment of skin and cartilage lesions (Galassi *et al.* 2000; Hollander AP, *et al.* 2006).
In the current study, progenitor cells were seeded into an HA biomaterial of non-woven mesh and cultures were supplemented with chondrogenic and osteogenic medium to develop bone and cartilage tissue *in vitro*. Time course of expression for the principal extracellular protein of bone and cartilage were analized and compaired.

Materials and Methods

Biomaterials

The biomaterial used in the present study was derived from the total esterification of hyaluronan (synthesized from 80-200 kDa sodium hyaluronate) with benzyl alcohol, and is referred to as HYAFF-11®. The final product is an uncross-linked linear polymer with an undetermined molecular weight; it is insoluble in aqueous solution yet spontaneously hydrolyzes over time, releasing benzyl alcohol and hyaluronan. HYAFF-11® was used to create non-woven meshes of 50 m-thick fibers, with a specific weight of 100 g/m^2. These devices were obtained from Fidia Advanced Biopolymers (FAB, Abano Terme, Italy).

Flow cytometric analysis

For flow cytometric analysis, the following phycoerythrin (PE)– and fluorescein isothiocyanate (FITC)–labeled mouse monoclonal antibodies and isotype negative controls were used: CD29-PE, CD166-PE, CD14-PE, CD34-PE, CD45-PE, SH2-PE, SH3-FITC, CD73 –PE and SH4–PE (DAKO, Glostrup, Denmark; Beckman Coulter, Miami, FL, USA). Cells were incubated with antibody for 15 minutes at room temperature for labelling, washed twice with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS. Flow cytometric analysis was performed with a FACScan (Becton Dickinson), for which settings and compensation were adjusted weekly by means of CaliBRITE beads (Becton Dickinson). The data were analyzed by CELLQuest and PAINT-A-GATE software (Becton Dickinson).

Cell cultures

Human/rat bone marrow mesenchyal stem cell (MSC) cultures
Bone marrow aspirates from human/inbred Fisher rat (Charles River Laboratories, Wilmington, MA, USA) femur were seeded on Petri dishes. After one day of culture, the medium was discarded and the adherent cell layer was washed twice and then cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. The media were changed twice a week and MSCs were allowed to grow until confluence. Cells were then trypsinized, tested for viability by eosin exclusion dye and finally seeded on HYAFF-11® three-dimensional scaffolds as described below.

Three-dimensional and monolayer cultures
Pieces (1×1 cm) of the HYAFF-11® non-woven material were fixed to culture plates with a fibrin clot and MSCs were seeded at a density of 5×10^5 cells/cm^2. MSC were seed onto Petri dishes (1 cm^2)at the same density. Culture media were supplemented with the following osteoblastic or chondrogenic factors:

Osteoblastic induction
DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 50 g/mL L-ascorbic acid (Sigma), 10 ng/mL fibroblast growth factor (FGF) (Calbiochem, CA, USA), dexamethasone 10 nM; β glycerophosphate 10 mM.

Chondrogenic induction
DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 50 g/mL L-ascorbic acid (Sigma), 1 ng/mL transforming growth factor-β1 (TGF-β1) (Calbiochem), 1 ng/mL of insulin (Sigma), 1 ng/mL epidermal growth factor (EGF), (Sigma) and 10 ng/mL basic fibroblast growth factor (EGF) (Sigma).

Table 1.

Primer	*Sequence*	*Size*
Human GAPDH	S TGGTATCGTGGAAGGACTCATGAC AS TGCCAGTGAGCTTCCCGTTCAGC	190
Human Osteocalcin	S ATGAGAGCCCTCACACTCCTC AS CTAGACCGGGCCGTAGAAGCG	303
Human Osteonectin	S ACATGGGTGGACACGG AS CCAACAGCCTAATGTGAA	405
Human Osteopontin	S CTTTCCAAAGTCAGCCGTGAATTC AS ACAGGGAGTTTCCATGAAGCCACA	532
Human Coll I	S GGTGGTTATGACTTTGGTTAC AS CAGGCGTGATGGCTTATTTGT	702
Human Coll II	S AACTGGCAAGCAAGGAGACA AS AGTTTCAGGTCTCTGCAGGT	621
Rat GAPDH	S GCCATCAACGACCCCTTCATT AS CGCCTGCTTCACCACCTTCTT	212
Rat Osteocalcin	S CAGCCCCCTACCCAGAT AS TGTGCCGTCCATACTTTC	232
Rat Osteonectin	S ACTGGCTCAAGAACGTCCTG AS GAGAGAATCCGGTACTGTGG	438
Rat Osteopontin	S CCAAGTAAGTCCAACGAAAG AS GGTGATGTCCTCGTCGTCTA	348

Table 2.

Amplification product	*Annealing T°*	*Time*	*Cycle*
Human GAPDH Human Coll I	62° C	60 sec	25
Human Osteocalcin Osteopontin Osteonectin	70 °C	60 sec	40
Human Coll II	65 °C	60 sec	40
Rat GAPDH	58 °C	60 sec	35
Rat Osteocalcin Osteopontin Osteonectin	58 °C	60 sec	40

After 3, 7, 14 and 21 days of culture, scaffolds and supernatants were separately collected and analysed for cell growth and differentiation.

In vitro proliferation of MSC cultures

To determine the kinetics of cell growth in monolayer and three-dimensional cultures, the MTT-based (Thiazolyl blue) cytotoxicity test was performed on days 3, 7, 14 and 21 according to the method of Denizot and Lang (Denizot *et al.* 1986) with minor modifications.

Electron microscopy

For ultrastructural evaluation, at day 21 three-dimensional osteogenic cultures were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 3 h, post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in araldite. Semithin sections were stained with toluidine blue and used for light microscopy analysis. Ultrathin sections were stained with uranyl acetate and lead citrate, and analyzed with a Philips EM400 electron microscope.

Immunohistochemical and histological analysis of three-dimensional cultures

Cryostatic sections (7 μm) of three-dimensional HYAFF-11® cultures were layered over gelatine-coated glass slides, fixed with absolute acetone for 10' at room temperature, and cryopreserved at 20°C until use. Type II collagen fibers present in the MSC-secreted extracellular matrix were visualized with the APAAP procedure (acid phosphatase anti-acid phosphatase). Briefly, after saturating non-specific antigen sites with 1:20 rabbit serum in 0,05M maleate TRIZMA (Sigma) pH 7,6 for 20', both 1:100 mouse anti-human/rat type II collagen (Sigma) were added to the samples. After incubation, samples were rinsed with buffer solution, and then second antibody was added for 30' (Link Ab-DAKO-, rabbit anti-mouse). After rinsing, sections were incubated for 30' with 1:50 mouse APAAP Ab-DAKO, rinsed again, and lastly, reacted for 20' with the Fast Red Substrate (Sigma). Counter staining was performed with haematoxylin (Sigma).

Real time RT-PCR

For each target gene, primers and probes were selected using Primer3 software. All primers are listed in Table 1. Gene expression was measured using real-time quantitative PCR on a Rotor-GeneTM 3500 (Corbett Research). PCR reactions were carried out using the primers at 300 nM and the SYBR Green I (Invitrogen) (using 2 mM MgCl2) with 40 cycles of 15 s at 95°C and 1 min at 60°C. All cDNA samples were analysed in duplicate. Fluorescence thresholds (Ct) were determined automatically by the software with efficiencies of amplification for the studied genes ranging between 92% and 110%. For each cDNA sample, the Ct value of the reference gene L30 was subtracted from the Ct value of the target sequence to obtain the ΔCt. The level of expression was then calculated as 2-ΔCt and expressed as the mean±SD of quadruplicate samples of two separate. Relative quanti-

tation of marker gene expression (Table 1) is given as a percentage of the beta actin product and the t-test was applied.

Statistical analysis

The one-way analysis of variance (Anova test) of the software package Excel (Microsoft office 2000) was used for data analyses. Repeat measurement analysis of variance (Re-ANOVA) and paired t tests were used to determine if there were significant ($p<0.05$) changes. Repeatability was calculated as the standard deviation of the difference between measurements of the test performed.

Results

Phenotypic characterization of human MSCs

Figure 1 illustrates the phenotypic characterization of culture-expanded human MSCs (hMSC) by flow cytometric analysis. Cells were consistently positive for β1 integrin (CD 29: 98.98%), CD 166 (95.86%), SH2 (93.22%), SH3 (96.63%) and SH4 (89.35%). Specific hematopoietic markers such as CD 14, CD 34 and CD 45 were consistently negative. Rat MSCs had a similar flow cytometric profile as humans: positivity for CD29; CD166; CD73 (*data not shown*)

MSC proliferation analysis

Figure 2 illustrates MSC growth in the presence of osteogenic differentiating medium in monolayer and three-dimensional conditions. Figure 2a shows that human cells proliferated and peaked as early as day 7. From day 14 to day 21, proliferation decreased and then stabilized at a lower plateau. Rat MSC proliferation peaked at day 15 of culture (Figure 2b). Comparing monolayer with 3D conditions is well evident, for both cell type, the positive effect of non woven on cell proliferation. The maintenance and proliferation of human and rat MSC onto the scaffold is confirmed by the higher MTT values. Indeed, in monolayer cells reach in 15 days confluence conditions showing a plateau in MTT value lower than 3D one where cells are able to growth in a bigger substrate eluding contact inhibition effect.

In Figure 3, the proliferation profile of human/rat MSCs cultured with chondrogenic differentiating medium is reported. Figure 3a illustrates human MSCs that had proliferated in three-dimensional and monolayer conditions, demonstrating the higher proliferation rate achieved in three-dimensional conditions, particularly in the latest stages of culture. A similar trend was observed in rat MSCs (Figure 3b), although the difference between three-dimensional and monolayer culture conditions was less evident than in human cells.

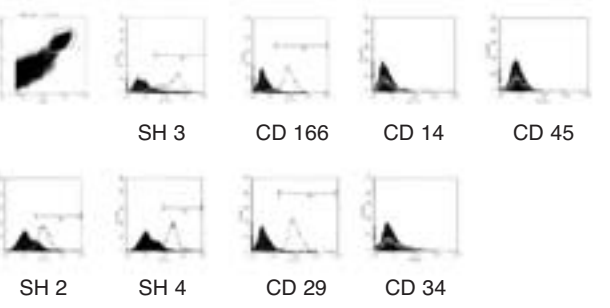

Figure 1. Cytofluorometric analysis of CD 29; CD 166; SH2; SH3; SH4; CD 14; CD 34; and CD 45. Solid profile represents cells stained with secondary antibody alone; Open profile represents cells stained with the anti CD 29; CD 166; SH2; SH3; SH4; CD 14; CD 34; and CD 45 antibody. Fibroblast are used as negative controls (*data not shown*). In each panel, the ordinate represents the number of cells. Data from an experiment representative of at least two similar experiments are shown.

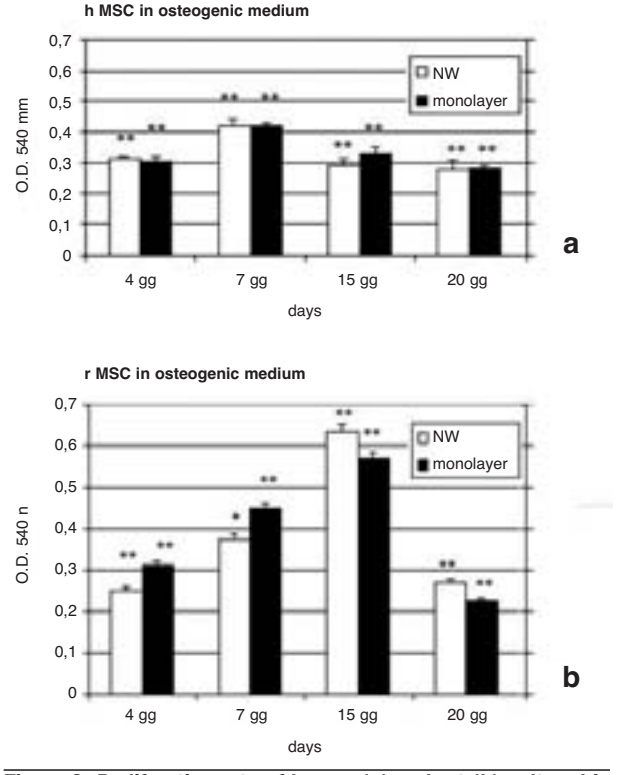

Figure 2. Proliferation rate of human (a) and rat (b) cultured in HYAFF11 ™ non-woven meshes (white bars, NW: non-woven) and in monolayer condition (black bars) in presence of osteogenic medium. The graphs represent the mean of three different experiments. Anova test: *$p<0.05$; **$p<0.01$.

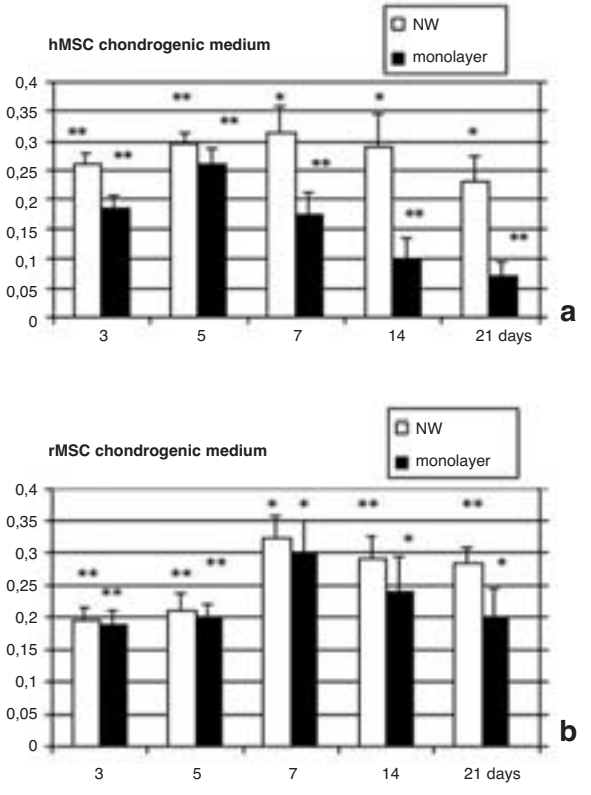

Figure 3. Proliferation rate of human (a) and rat (b) cultured in HYAFF11™ non-woven meshes (white bars, NW: non-vowen) and in monolayer condition (black bars) in presence of chondrogenic medium. The graphs represent the mean of three different experiments. Anova test: *p<0.05; **p<0.01.

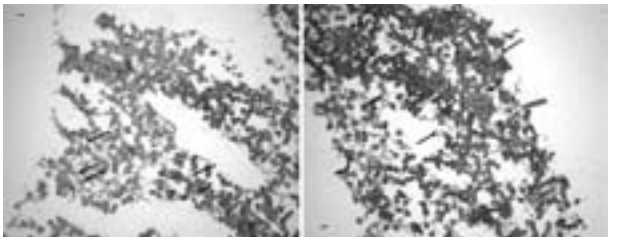

Figure 4. Immunolocalization of type-II collagen in cryostatic section of human (a) and rat (b) MSC after 21 days of culture in 3D cultures in presence of chondrogenic medium. Collagen (black arrows) was present both within the biomaterial interstices and around the biomaterial fibers (white arrows) (X20). Bar: 50 µm.

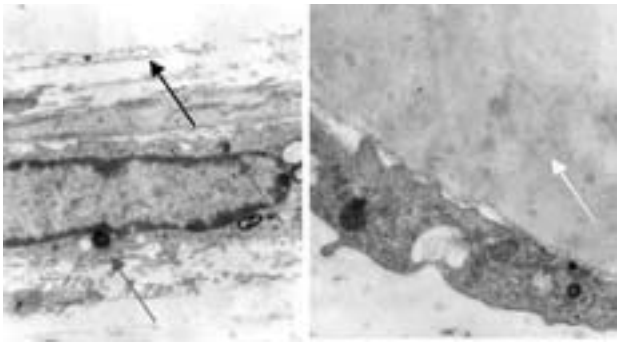

Figure 5. Electron microscopy of hMSC cultured on Hyaff® 11 for 21 days. Cells cultured in osteogenic medium. Some matrix vesicles (grey arrows) are visible in the extracellular matrix close to partially calcified collagen fibres (black arrow). Biomaterials fibers are indicated by yellow arrow. Magnification: (a)= 4600.

Histological and immunohistochemical analysis

Chondrocyte differentiation

Figure 4 illustrates the immunostaining of collagen type II secreted in three-dimensional cultures of both human (Figure 4a) and rat (Figure 4b) MSCs after 21 days. Collagen fibres (black arrows) were present inside the scaffold interstices and the cells filled the inner non-woven fibers (white arrows). A very faint immunostaining reaction for type II collagen was detectable in cells cultured in monolayer (*data not shown*).

Electron microscopy analysis

Electron microscopic analysis of human MSCs in three-dimensional culture (Figure 5) revealed a typical osteoblastic phenotype: a large ovoid nucleus and extensive granular endoplasmic reticulum. Figure 5 a/b illustrates a mineralized area with matrix vesicles in the extracellular spaces close to partly calcified collagen fibres. These cells, which contained a large amount of granular endoplasmic reticulum, were completely surrounded by fully mineralized bone matrix. No significant differences were found between human and rat MSC cultures.

Real time rtPCR

rT-PCR was performed on MSC cultures in monolayer and three-dimensional scaffolds to monitor at the mRNA level cell differentiation in the presence of chondrogenic/osteoblastic medium. Total RNA samples were extracted after 7, 14, 21, 28, 35 days of culture and the expression of chondrogenic/osteoblastic marker genes such as type I and II collagen, osteopontin, osteocalcin, osteonectin was determined. Values are reported as gene/β actin level.

Chondrocyte differentiation

As reported in Figure 6a, collagen type I expression in human and rat MSCs in three-dimensional scaffolds showed a progressive decrease over time. Conversely, collagen type II (Figure 6b) progressively increased in both cell types, peaking at day 21. In monolayer culture of both cell types,

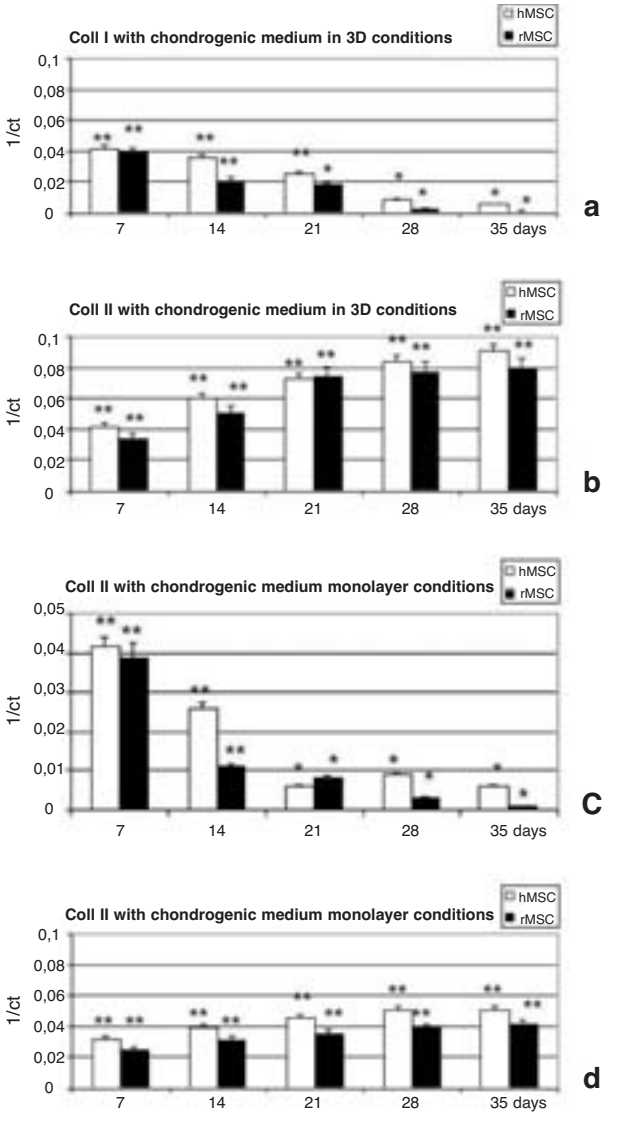

Figure 6. Time course of: collagen I mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (c) in presence of chondrogenic medium. Collagen II mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (b) and in monolayer condition (d) chondrogenic medium.

collagen type I was consistently expressed over time (Figure 6c), while type II collagen was weakly expressed (Figure 6d) and tended to decrease over time.

Osteocyte differentiation

Figure 7a illustrates the expression of collagen type I in human and rat MSCs cultured in three-dimensional scaffolds. Collagen I mRNA production peaked at day 14 and after a temporary drop off at day 21, progressively increased. Figure 7b illustrates the comparatively lower expression of collagen type I in human and rat MSCs cultured in monolayer conditions.

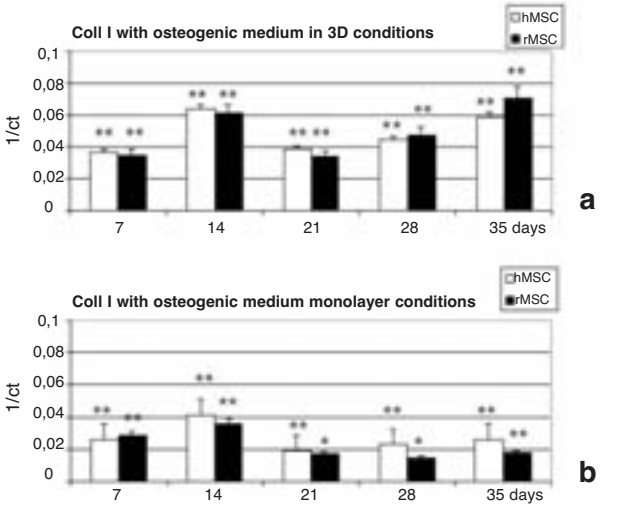

Figure 7. Time course of: collagen I mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of osteogenic medium.

Figure 8a illustrates the expression of osteocalcin, Figure 9a of osteopontin and Figure 10a of osteonectin in human and rat MSCs both in three-dimensional and in monolayer conditions. Osteocalcin expression was similar in both cell types and increase over time. Osteopontin expression was greater than osteocalcin during and appeared constant over time. Osteonectin expression showed a progressive decrease over time for both cell types. In monolayer culture, osteocalcin, osteopontin and osteonectin expression was comparatively lower, but demonstrated the same trend as in three-dimensional cultures (Figures 8/9/10b).

Discussion

In vitro tissue replacement of bone and cartilage has long been a conundrum to be solved by clinicians and tissue engineers. Developments in therapeutic strategies on cartilage repair have increasingly focused on the promising technology of cell-assisted repair proposing to used autologous chondrocytes or other cell types to regenerate articular cartilage *in situ*. The necessary requisites include the correct cell type and ideal degradable and biocompatible 3D scaffold with favourable structural features for cell attachment, proliferation, chondrogenesis and osteogenesis *in vitro* and functional integration *in vivo*. As regard to biomaterial, hyaluronan based scaffolds, such as HYAFF11, are biodegradable materials currently used for tissue engineering of skin and cartilage. This

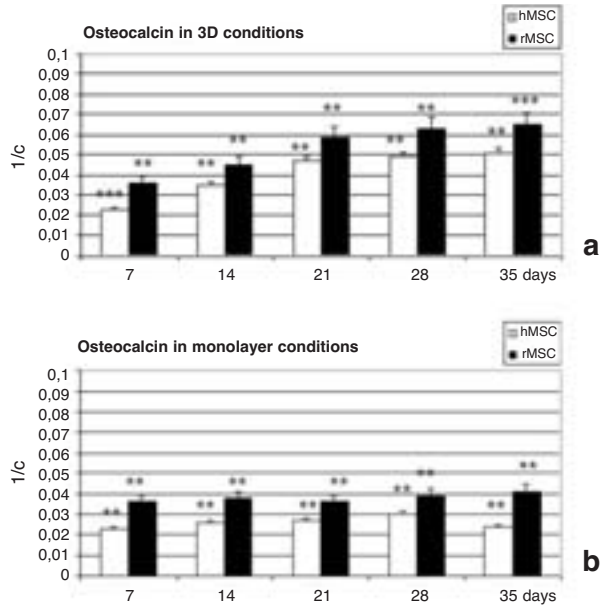

Figure 8. Time course of osteocalcin mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of chondrogenic medium.

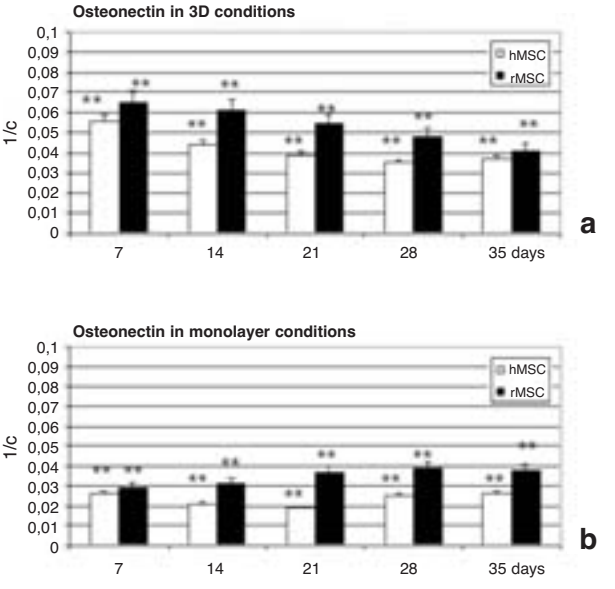

Figure 10. Time course of osteopontin mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of chondrogenic medium.

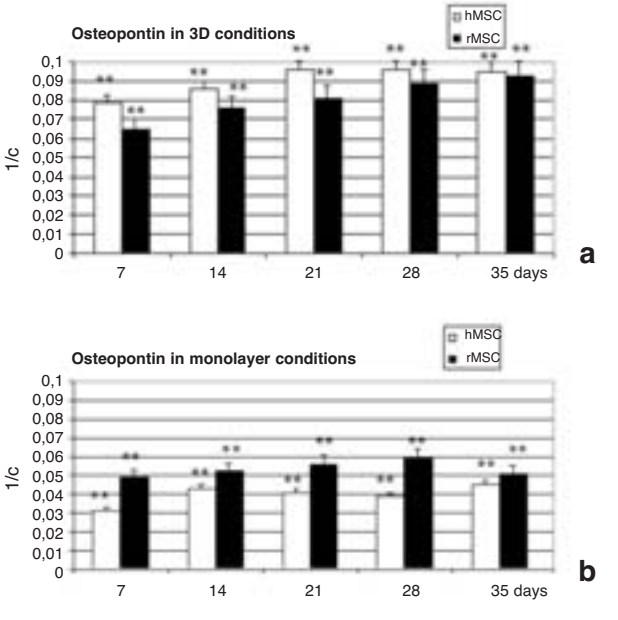

Figure 9. Time course of osteopontin mRNA expression analyzed by semi-quantitative RT-PCR in hMSC (white bars) and rMSC (black bars) cultured on HYAFF® -11 (a) and in monolayer condition (b) in presence of chondrogenic medium.

material is highly compatible with cells and matrix and its degradation products induce extracellular matrix production and neoformation of blood capillaries (Tonello *et al.* 2005).

In autologous cell implantation a currently practiced cell-based therapy to repair cartilage defects, autologous chondrocytes are recovered from the patient but are considered too sparse for direct re-implantation. To overcome cell scarcity, chondrocytes are amplified in tissue culture prior to re-implantation, but after at least four doublings, chondrocytes can non longer produce cartilage matrix. In contrast to adult chondrocytes, MSC are easier to obtain and can be manipulated for multiple passages. MSC-based cartilage repair had been attempted in animal models but is still at the early stage of clinical trial for applications in human. MSCs are currently the most promising source for *in vitro* and *in vivo* reconstruction of new hard connective tissue such as bone and cartilage. Indeed, the presently reported data confirm that bone marrow MSCs can be isolated and cultured both in monolayer and in three-dimensional conditions in the presence of chondrogenic/osteogenic medium. Cytofluorimetry confirmed that isolated MSCs from human and rat bone marrow are natural progenitors since they possess the most common specific markers. From the analysis of the principal surface antigens, cells appeared consistently non-hematopoietic and non-endothelial since they were negative for the hallmark antigens of the hematopoietic stem cell such as CD14, CD45, CD34 (Gronthos S, *et al.* 2003). Conversely, they expressed the typical mesenchymal cell markers such as CD29 (anti β1 integrin), SH-2 (recognizing the transmembrane glycoprotein endoglin: CD 105), SH-3 and SH-4 (recognizing CD73) for hMSC and CD73 for

rMSC (Haynesworth SE, *et al.* 1992). After expansion in monolayer culture and in the presence of chondrogenic and osteogenic inducing factors, human and rat MSCs differentiated into chondrocytes and osteoblasts, respectively. When cultured in osteogenic conditions, the proliferation rate of MSCs increased during the initial period of culture, progressively decreasing after differentiation both in 3D and in monolayer conditons. Detailed rtPCR analyses of extracellular matrix components (collagen type I, osteopontin, osteocalcin and osteonectin) confirmed the presence of osteogenic molecules already after one week of monolayer or three-dimensional culture. In particular, in this early phase of osteogenic differentiation high levels of osteonectin, a molecule fundamentally important for cellular-bone matrix interaction and for matrix mineralization, were observed in 3D conditions. Collagen type I molecules, essential for formation and maturation of hydroxyapatite crystals, were also detected during the first 10 days of culture. Light and electron microscopy of three-dimensional cultures of MSCs in osteogenic medium demonstrated a well organized extracellular matrix in which type I collagen fibres and calcium phosphate crystals were co-localized. Interestingly, both cell proliferation and expression of human and rat MSCs were consistently higher in osteogenic cells in three-dimensional versus monolayer culture. The three-dimensional hyaluronan scaffolds permitted differentiation of MSCs to a chondrogenic phenotype as well. Time dependent increases in cell proliferation were greater in three-dimensional compared to monolayer culture conditions. These are similar findings to those observed with adult chondrocytes (Brun *et al.* 1999). The expression and production of collagen type II, a well-documented marker of hyaline articular cartilage always found in freshly isolated chondrocytes, was determined by molecular expression and (rtPCR) morphological analyses. Findings again confirmed that the chondrogenic differentiation process was better promoted in three-dimensional culture than in monolayer. Conversely, collagen type I was expressed in three-dimensional culture predominately during the initial phases of the differentiating process, while in monolayer conditions it increased progressively over time. Although human and rat MSCs have the same diferentiating potential, they do behave differently during the proliferation process. While human cell proliferation peaks after one week of culture, rat cell proliferation peaks after two weeks. These results demonstrate that both human and rat MSCs can be cultured in three-dimensional scaffolds made from hyaluronan based polymers in the presence of the necessary stimuli that support differentiation towards osteogenic or chondrogenic phenotypes. The delivery vehicles investigated in this study are easily applicable to clinical practice since hyaluronan scaffolds have been already extensively studied both for the *in vitro* reconstruction of skin and cartilage substitutes and for their clinical application. In the end, these data clearly confirm that bone marrow cells are progenitor cells that are clearly superior to tissue biopsy-isolated cells for use in tissue engineering. Tissue samples from patients have to be isolated by enzymes such as collagenase and hyaluronidase to remove extracellular matrix components and, as is well known, adult stem cells usually are very scarcely supplied within tissues. MSCs isolated from the bone marrow would be a valuable source for cell transplantation since their characteristic features include a high potential for proliferation and multilineage differentiation.

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Tendon crimps and peritendinous tissues responding to tensional forces

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Tendons transmit forces generated from muscle to bone making joint movements possible. Tendon collagen has a complex supramolecular structure forming many hierarchical levels of association; its main functional unit is the collagen fibril forming fibers and fascicles. Since tendons are enclosed by loose connective sheaths in continuity with muscle sheaths, it is likely that tendon sheaths could play a role in absorbing/transmitting the forces created by muscle contraction.
In this study rat Achilles tendons were passively stretched *in vivo* to be observed at polarized light microscope (PLM), scanning electron microscope (SEM) and transmission electron microscope (TEM). At PLM tendon collagen fibers in relaxed rat Achilles tendons ran straight and parallel, showing a periodic crimp pattern. Similarly tendon sheaths showed apparent crimps. At higher magnification SEM and TEM revealed that in each tendon crimp large and heterogeneous collagen fibrils running straight and parallel suddenly changed their direction undergoing localized and variable modifications. These fibril modifications were named *fibrillar crimps*. Tendon sheaths displayed small and uniform fibrils running parallel with a wavy course without any ultrastructural aspects of crimp. Since in passively stretched Achilles tendons fibrillar crimps were still observed, it is likely that during the tendon stretching, and presumably during the tendon elongation in muscle contraction, the fibrillar crimp may be the real structural component of the tendon crimp acting as shock absorber. The peritendinous sheath can be stretched as tendon, but is not actively involved in the mechanism of shock absorber as the fibrillar crimp. The different functional behaviour of tendons and sheaths may be due to the different structural and molecular arrangement of their fibrils.

Key words: Achilles tendon, sheaths, collagen fibrils, TEM, SEM.

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Joint movements of the body in mammals are generated by skeletal muscle cell activity, but the structures of the muscle-tendon complex able to transmit the forces of muscle contraction to bone are tendons and aponeuroses (Magnusson *et al.,* 2003). Tendons are considered highly flexible but inextensible structures offering a considerable resistance to tension. They also act as mechanical buffers or shock absorbers in protecting tendons to bone attachment during the initial elongation related to rapid muscle contraction (Stolinski, 1995a).

Tendons are dense fibrous collagen structures organized in a hierarchical manner whose main functional unit, strong and stiff in tension, is the collagen fibril (Kannus, 2000; Provenzano and Vanderby, 2006). The particular arrangement and dimensions of the collagen fibrils, together with their interactions with hydrophilic proteoglycans of the extracellular matrix, are responsible for the transmission of forces and resistance to tension. Collagen fibrils run straight and parallel in relaxed tendons, and are always arranged in fibers, fibril bundles and fascicles showing a zig-zag or waveform aspect called *crimping*. During initial stretching the crimps disappear or become more flattened acting as shock absorbers to tension (Diamant *et al.,* 1972; Kastelic *et al.,* 1980; Screen *et al.,* 2004; Franchi *et al.,* 2007). Increasing the tensile strength, the intra- and intermolecular cross-links of collagen fibrils are primarily involved in the transmission of mechanical forces (Kjaer, 2004; Provenzano and Vanderby, 2006). During this phase proteoglycans with their bridges also play a role in absorbing and/or transmitting the tension stress to bone (Cribb and Scott, 1995; Fratzl *et al.,* 1998; Scott, 2003).

Tendons are often surrounded by loose connective sheaths forming the paratenon, epitenon, peritenon and endotenon (Strocchi *et al.,* 1985; Kannus, 2000; Kjaer, 2004). According to Trotter and Purslow (1992) and Kjaer (2004) tendon sheaths

are linked to skeletal muscle sheaths and it is reasonable to think that even these apparently indifferent membranes play a role in absorbing and/or transmitting tensional forces in tendon.

Microscopic and ultrastructural analyses of rat tendons in this study may shed light on the morphologic and functional changes to collagen in tendon and peritendinous tissues when tendon is mechanically stretched *in vivo*.

Materials and Methods

Animals

Twelve female Sprague-Dawley rats (3 months old) were anaesthetized with an intraperitoneal injection of 87 mg/kg ketamine (Ketavet, Farmaceutici Gellini Spa, Italy) and 13 mg/kg xylazine (Rompun, Bayer Italia Spa, Italy). A resin brace, modified to induce foot dorsal flexion, was applied to one posterior leg in order to reach a final 55° angle flexion.

The stretching position was kept for 10 minutes. At the end of the stretching session and still under anaesthesia, the tendon of the gastrocnemius muscle with its sheaths was exposed and fixed in situ (i.e. still connected to the muscle belly and to the bone) in Karnovsky's solution. The tendon of the controlateral leg of each animal was kept relaxed and fixed as with the stretched tendon to be analysed as a control sample. Finally, the rats were euthanized via an intracardiac injection of Tanax (Hoechst, Frankfurt am Main, Germany).

All stretched and control tendons with their own sheaths were excised. Ten tendons (five stretched and five controls) were processed for polarized light microscopy (PLM). The other eight tendons (four stretched and four controls) were processed for transmission electron microscopy (TEM) and the last six tendons (three stretched and three controls) were longitudinally cut to be investigated by scanning electron microscopy (SEM).

The experimental protocols were conducted in accordance with Italian and European Laws on laboratory animals use and care.

Polarized light microscopy

Specimens were fixed in 10% buffered formalin, dehydrated in graded concentrations of ethanol, embedded in paraffin and longitudinally sectioned at 6 μm. The sections were stained with 5% Picrosirius Red to enhance the natural birifrangence of collagen fibers when observed under the polarized light microscope (Leitz Ortholux 2, Wetzlar, Germany).

Transmission electron microscopy

Specimens for TEM were fixed in Karnovsky' s solution, rinsed with a 0.1M sodium cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide. Thereafter, they were dehydrated in graded alcohols and embedded in Araldite resin. The ultrathin sections were stained with lead citrate and uranyl acetate and viewed under a Philips CM-10 electron microscope.

Scanning electron microscopy

For SEM study, the samples were fixed in Karnovsky's solution, dehydrated in a graded ethanol series and then in hexamethyldisilazane. Finally they were mounted on metal stubs and coated with gold using a sputter coater (Emitech K550). Observations were made under SEM (Philips 515 and Philips XL30-FEG) operating in secondary-electron mode.

Results

Relaxed Achilles tendon

Longitudinal sections of relaxed rat Achilles tendon analyzed by light microscopy showed parallel collagen fibers with a wavy course that under polarized light microscope is displayed as alternating dark and light bands corresponding to *tendon crimps* (Figure 1). Flat fibroblast-like cells were interposed between adjacent fiber bundles. The outer surface of the Achilles tendon was covered by a sheath of collagen fiber bundles running in a waveform pattern. At the polarized light microscope the collagen fibers of this sheath showed dark and light bands similar to the tendon crimps (Figure 1).

Other specimens observed at SEM showed the tendon fibers to be composed of large plurimodal collagen fibrils running straight and parallel. At the crimp apex these fibrils suddenly changed their direction showing an evident elbow with knots corresponding to deformations of the fibril shape. In particular, collagen fibrils appeared bent on the same plane like bayonets, or twisted and bent (Raspanti *et al.,* 2005; Franchi *et al.,* 2007) (Figure 2). The tendon sheath appeared composed of thin wavy collagen fibers made up of small unimodal collagen fibrils. No crimps were recognizable

along these fibril bundles (Figure 3).

Other specimens analysed at TEM better showed that tendon collagen fibrils, when changing their direction at the crimp apex, modified their shape (bent on the same plane like bayonets, or twisted and bent) and lost their D-period disclosing their microfibrillar arrangement (Figure 4). As in previous SEM observations, thin sections showed the small collagen fibrils of the sheaths running in a smooth undulating arrangement without any ultrastructural aspects of crimp (Figure 5).

Stretched Achilles tendon

Longitudinal sections of stretched rat Achilles tendons observed at direct and polarized light microscope showed most of the tendon collagen fibers running straight and parallel with a few flattened crimps (Figure 6). The collagen fibers in stretched tendon sheaths ran straight with a slightly wavy course.

In similar specimens observed at SEM tendon fibers showed rare or otherwise completely flattened crimps. In all crimps, including those whose collagen fibrils appeared completely straightened, the fibrils still retained the knots at the apex of the crimps as in relaxed specimens (Figure 7). On the contrary tendon sheath collagen fibrils showed a less undulating path than the relaxed specimens and no ultrastructural knot or fibril size deformation was detectable at ultrastructural level (Figure 8).

At TEM, the same fibril knot described in relaxed specimens were detected even in straightened fibrils of completely flattened crimps (Figure 9). Collagen fibrils of fiber bundles in tendon sheath appeared partially stretched along the main axis of tendon (Figure 10).

Discussion

A waveform configuration of collagen fibers in tendon was first described in polarized light microscopy investigations. The authors correlated the periodic crimping pattern to tendon functions observing that crimping disappeared when tendons were slightly stretched *in vitro* (Rigby *et al.,* 1959; Elliot, 1965; Viidik and Ekholm, 1968; Stromberg and Wiederhielm, 1969; Viidik, 1972; Hess *et al.,* 1989). Some authors (Diamant *et al.,* 1972; Atkinson *et al.,* 1999; Hansen *et al.,* 2002) suggested that the alignment of collagen fibers during stretching of the tendon might correspond to the toe region of the stress-strain curve of tendon. Ultrastructural studies were also carried out to improve the morphological or functional meaning of tendon crimps, but no new functional data were reported (Rowe, 1985a,b; Gathercole and Keller, 1991; Stolinski, 1995a; Magnusson *et al.,* 2002; Hurschler *et al.,* 2003). Recently Franchi *et al.* (2007) described a morphological deformation of collagen fibrils in tendon crimps and named it *fibrillar crimp*. They also observed that fibrillar crimps did not disappear when the Achilles tendon was physiologically stretched *in vivo*, suggesting a modification of the fibril structure at the level of fibrillar crimps.

The study of tendon stretching may help to shed light on the mechanism of force transmission during muscle contraction.

According to Kjaer (2004) tendon sheaths are in continuity with the peri- and intra-muscular collagen sheaths thereby ensuring a functional link between the skeletal muscle and bone. In particular the perimysium seems to play a role in transmitting tensile force (Trotter and Purslow, 1992). It has been suggested that the connective tissue of skeletal muscle and tendon is like a lively structure with a dynamic protein turnover, highly able to adapt to changes in the external environment such as mechanical loading or inactivity and disuse (Kjaer, 2004). As tendon is tightly connected to the skeletal muscle via connective tissue of tendon and muscle sheaths it is probable that the peritendinous collagen fibers might be involved in transmission of forces from muscle to tendon.

Morphological flattened waves of collagen fibers comparable to those described in tendons were also observed in nerve sheath as in the epineurium (Stolinski, 1995b). The pattern was observed in cut or relaxed fascicles *in situ* as well as in isolated and split layers of the nerve sheath. It is interesting that the pattern was not observed on nerve fascicles under tension. The nature of the wavy structure suggested that the sheath length might change on stretching or contraction to accommodate the displacement and movement of nerve fibres (Stolinski, 1995b).

At polarized light microscope the present study disclosed a waveform pattern of collagen fibers both in tendon and tendon sheaths. However, while the waveform pattern of tendon crimps is due to a peculiar structural characteristic of the collagen fibrils (a structure specifically acting as a shock absorber and named *fibrillar crimp*), the waveform

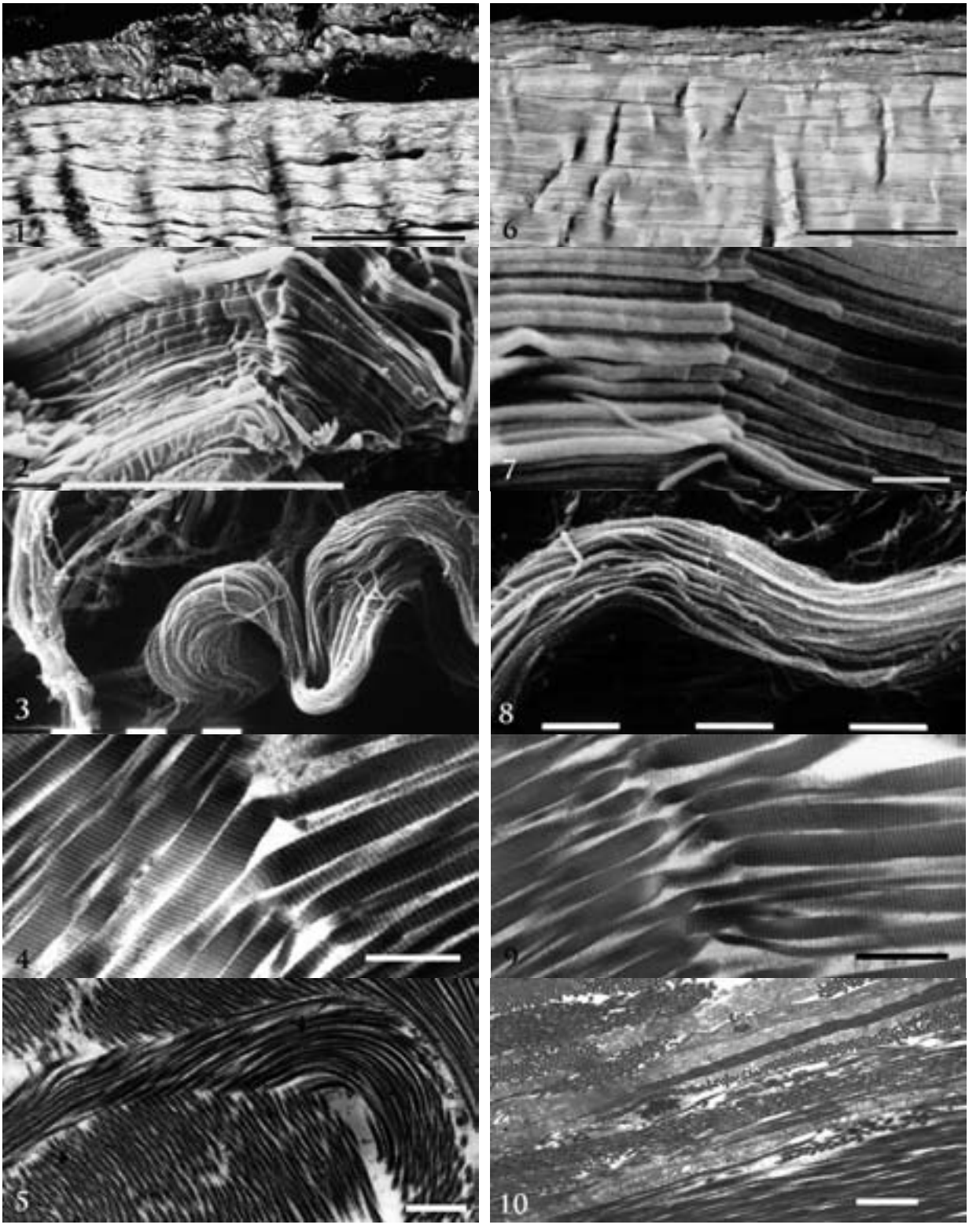

Figure 1. Relaxed rat Achilles tendons at PLM. Crimped fibers of tendon sheath (top) and crimped tendon fibers (bottom). Scale bar = 100 µm. Figure 2. Relaxed rat Achilles tendons at SEM. Fibrillar crimps in a tendon crimp. Scale bar = 10 µm. Figure 3. Relaxed rat Achilles tendons at SEM. Undulating fibrils in a tendon sheath fiber. Scale bar = 1 µm. Figure 4. Relaxed rat Achilles tendons at TEM. Fibrillar crimps in a tendon crimp. Scale bar = 1 µm. Figure 5. Relaxed rat Achilles tendons at TEM. Undulating collagen fibrils of tendon sheath. Scale bar = 100 µm. Figure 6. Stretched rat Achilles tendons at PLM. Straightened tendon sheath (top) and straightened tendon fibers (bottom). Scale bar = 100 µm. Figure 7. Stretched rat Achilles tendons at SEM. Fibrillar crimps in straight fibrils. Scale bar = 1 µm. Figure 8. Stretched rat Achilles tendons at SEM. Straightened fibrils of tendon sheath. Scale bar = 1 µm. Figure 9. Stretched rat Achilles tendons at TEM. Fibrillar crimps in straight fibrils. Scale bar = 1 µm. Figure 10. Stretched rat Achilles tendons at TEM. Straightened fibrils in tendon sheath. Scale bar = 100 µm.

configuration of tendon sheath appears as a simple undulating arrangement of collagen fibrils with no fibrillar crimps. Therefore, the straightening of the sheath collagen fibrils should be interpreted as a passive morphological adaptation to changes in tendon length.

Transmission of forces from skeletal muscle to bone involves different phases in tendon elongation. During initial tendon stretching crimps disappear or become more flattened acting as shock absorbers to tension with no local tissue strain increase (Diamant *et al.,* 1972; Kastelic *et al.,* 1980; Screen *et al.,* 2004; Franchi *et al.,* 2007). Increasing the tensile strength, the intra- and intermolecular cross-links of collagen fibrils are then involved in the transmission of mechanical forces (Kjaer, 2004; Provenzano and Vanderby, 2006). Some authors suggest that short proteoglycan bridges linked to collagen fibrils, like decorin, may also absorb and then transmit the tension stress to bone (Cribb and Scott, 1995; Fratzl *et al.,* 1998; Scott, 2003). Our results may suggest that during the passive static stretching of tendon, and presumably during tendon elongation in muscle contraction, the peritendinous sheath can be stretched like tendon, but is not actively involved in the shock absorber mechanism like the fibrillar crimp. The different functional behaviour of these two structures (tendons and sheaths) is also due to the different structural and molecular arrangement of the fibrils: tendon fibrils are large in diameter, parallely tightly packed and with a straight microfibrillar arrangement; fibrils in tendon sheaths are small and uniform in diameter, run in thin wavy bundles and have an helicoidal microfibrillar arrangement. Attending to the distribution in the connective tissue of the body, tendons are prevalently submitted to unidirectional tensional forces while sheaths undergo multidirectional loading (Ottani *et al.,* 2001).

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The mechanism of transduction of mechanical strains into biological signals at the bone cellular level

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As appears from the literature, the majority of bone researchers consider osteoblasts and osteoclasts the only very important bony cells. In the present report we provide evidence, based on personal morphofunctional investigations, that such a view is incorrect and misleading. Indeed osteoblasts and osteoclasts undoubtedly are the only bone forming and bone reabsorbing cells, but they are transient cells, thus they cannot be the first to be involved in sensing both mechanical and non-mechanical agents which control bone modeling and remodeling processes. Briefly, according to our view, osteoblasts and osteoclasts represent the *arms of a worker*; the actual *operation center* is constituted by the cells of the osteogenic lineage in the resting state. Such a resting phase is characterized by osteocytes, bone lining cells and stromal cells, all connected in a functional syncytium by gap junctions, which extends from the bone to the vessels. We named this syncytium the *Bone Basic Cellular System* (BBCS), because it represents the only permanent cellular background capable first of sensing mechanical strains and biochemical factors and then of triggering and driving both processes of bone formation and bone resorption. As shown by our studies, signalling throughout BBCS can occur by *volume transmission* (VT) and/or *wiring transmission* (WT). VT corresponds to the routes followed by soluble substances (hormones, cytokines etc.), whereas WT represents the diffusion of ionic currents along cytoplasmic processes in a neuron-like manner. It is likely that non-mechanical agents first affect stromal cells and diffuse by VT to reach the other cells of BBCS, whereas mechanical agents are first sensed by osteocytes and then issued throughout BBCS by WT.

Key words: osteogenic cells, osteoclasts, cytokines, mechanical strains.

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It is a well established fact that, under the control of mechanical agents (body weight, force of gravity, muscular tone and strength) and non-mechanical agents (hormones, vitamins, cytokines, growth factors), bone cells regulate bone homeostasis and take part in the maintenance of mineral homeostasis, by means of three processes: bone growth, bone modeling and bone remodeling. Bone growth and bone modeling are only devoted to the regulation of bone homeostasis, whereas bone remodeling takes part in the regulation of bone homeostasis as well as of mineral homeostasis, by respectively improving bone structure in response to mechanical demands and setting free calcium and phosphate ions during the reabsorbing phase.

Frost's mechanostat theory (Frost, 1987) and Utah paradigm (1985) have greatly rationalized bone modeling and remodeling processes and what they involve at the bone macroscopic level. However what happens at the cellular level still remains to be defined. We do not know, for instance: a) how mechanical agents and non-mechanical agents interact at the cellular level; b) which is the mechanism of transduction of mechanical strains into biological signals; updated literature ascribes to osteocytes the function of sensing the strains induced into the bone matrix by mechanical stresses but, as we will discuss below, all cells of the osteogenic system are likely to be affected by mechanical strains; c) how osteocytes transmit mechanical stimuli to, and interact with, the other bone cells.

In the attempt to answer these questions we will first summarize the results of the morphofunctional investigations we carried out on the cells of the osteogenic lineage during the last three decades. Then we will discuss some functional implications.

The cells of the osteogenic lineage: morphological aspect and function

In the early 1970s, we showed that the exponential decrement of the appositional growth rate,

which has been shown to occur during osteon formation by means of triple fluorochrome technique (Manson and Waters, 1963; Marotti and Camosso, 1968), depends on the diminution in size of the osteoblasts and their progressive flattening. At the beginning of osteon formation, when the appositional rate is high, the osteoblasts are big and prismatic, whereas towards the end of osteon formation, when the rate is low, they are smaller and flat (Marotti, 1976).

Since these facts were also observed in trabecular bone, our conclusion was that the rate at which the bone tissue is laid down depends on the ratio between the *volume* of the osteoblasts and their secretory territory: the greater the osteoblast volume and the smaller its *secretory territory*, the higher the rate of bone apposition (Marotti, 1976).

Additionally we showed that, during the edification of osteons, also the osteocytes decrease in size, in parallel to the decrement of osteoblast dimension and the appositional growth rate. This finding implies that the size of the osteocytes strictly depends on the size of the osteoblasts from which they differentiate: the bigger the osteoblasts the larger the size of the osteocytes (Marotti, 1976). The functional meaning of this fact as yet to be established. However we found, in human osteons, that the decrement in size of osteocytes from the cement line towards the Haversian canal is paralleled by a thinning of *osteocytic-loose* (collagen poor) lamellae and, consequently, by a diminution of the distance between *non-osteocytic-dense* (collagen rich) *lamellae,* whose thickness does not significantly change throughout the osteonic wall. Mechanically speaking, this fact involves an increase in collagen fibers, namely in bone strength, along the bone surfaces where stresses and strains reach the highest values (Ardizzoni *et al.,* 1999).

In more recent years, we showed by transmission and scanning electron microscopes that the arborization of osteocytes is asymmetrical as regards both number and length of cytoplasmic processes. Vascular dendrites (those radiating toward the bone vascular surface) are more numerous (Marotti *et al.,* 1985) and incomparably longer than mineral dendrites (those radiating towards the opposite surface) (Palumbo, 1986; Palumbo *et al.,* 1990a, 1990b). Therefore osteocyte appear to be polarized cells, towards the bone surface where they come into contact whether osteoblasts or bone lining cells, according to which the bone surface is growing or resting.

Additionally we found that the number of osteocyte vascular dendrites coming into contact with each osteoblast is inversely proportional to the osteoblast size, namely to its bone forming activity. This fact suggests a possible inhibitory effect of osteocytes on osteoblasts (Marotti *et al.,* 1992).

In subsequent series of transmission electron microscope investigations we found that also bone-associated stromal cells are dendritic elements. They form a continuous cytoplasmic network which extends from endothelial cells to bone lining cells or osteoblasts (Palazzini *et al.,* 1998). Since gap junctions (actually considered as electrical synapses, when active) were observed throughout all cells of the osteogenic system, including stromal cells, it seems likely that not only osteocytes but all cells of the osteogenic lineage are functionally connected in a syncytium.

On the basis of these findings, we postulated that the transmission of signals throughout the cells of the osteogenic system may occur by means of two mechanisms: *volume transmission* (VT) and *wiring transmission* (WT). VT corresponds to the well-known routes followed by hormones, cytokines and growth factors to reach the bone cells. The novelty of our hypothesis lies in the suggestion that the cells of the osteogenic lineage may communicate reciprocally and modulate their activity by WT, namely in a neuron-like manner (Marotti *et al.* 1993, 1996; Marotti, 1996). Indeed some similarities do exist between osteocytes and neurons. Mineral cytoplasmic processes of osteocytes resemble neuronal dendrites in that they are shorter, thicker and may contain cell organelles, whereas osteocyte vascular cytoplasmic processes are longer, slender and do not contain organelles, thus resembling neuronal axons. Transmission of signals through osteocytes seems to occur by gap junctions instead of synapses, though it has been shown that osteocytes produce typical neurotransmitters like nitric oxide (Zaman *et al.,* 1999) and amino acid glutamate (Skerry, 1999).

In recent years we provide evidence that WT really occurs along osteocytes in amphibian (Rubinacci *et al.,* 1998) as well as in murine (Rubinacci *et al.,* 2002) cortical bone. Metatarsal bones, placed in an experimental chamber in *ex vivo* conditions, were subjected by a mechanical stimulator to pulsing axial loading by varying the loading parameters: amplitude and frequency. A 200 micra hole was

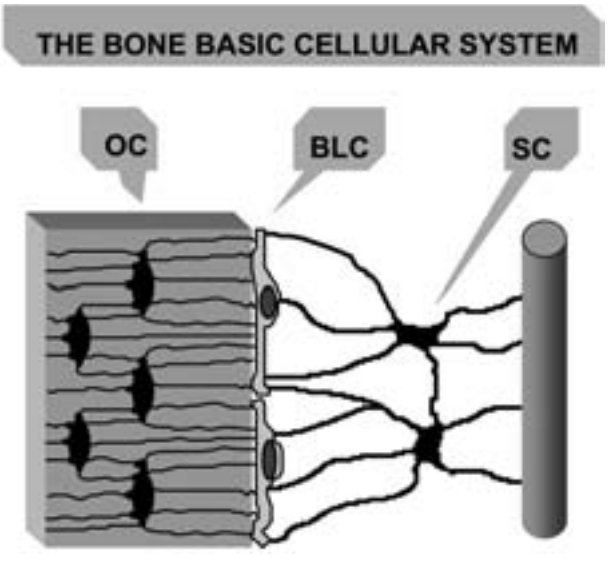

Figure 1. Schematic drawing of the cells of the osteogenic lineage in the resting phase, the so called *Bone Basic Cellular System*. From left to right: osteocytes (OC), bone lining cells (BLC), stromal cells (SC) and a vascular capillary. This network of cells forms a functional syncytium since they are all joined by *gap junctions*. It is suggested that this syncytium is capable of sensing both mechanical strains and biochemical factors and, at any moment, after having combined the two types of stimuli, it issues by *wiring* and/or *volume transmission* the appropriate signals that activate bone formation or bone resorption.

previously drilled through the metatarsal cortex and the ionic currents entering the hole were monitored by a two-dimensional vibrating probe system.

The following results were obtained. *Before loading*: signal of 15.5±4.6 micronA/cm^2 was recorded for living bone; no signal was detected for dead bone (i.e. dead osteocytes). *After loading under 5 g at 1Herz*: a) dead bone, too, exhibited an ionic current, but living bone drove a current about 4 times higher; b) the time pattern decay in dead bone tended linearly to 0 within 70′; in living bone it decreased exponentially, approaching the basal values within 15′ and afterwards it remained steady over time. By increasing the load from 0.7 to 12 g at a fixed frequency of 1Hz, the current increased with increasing loads up to 8 g only, but under higher loads it persisted at a higher level over time. By increasing the frequencies from static to 2Hz at a fixed load of 5 g, we recorded the same results obtained by increasing the loads at a constant frequency. Static load did not induce any current. Briefly, these findings indicate that: 1) bone strains induce an ionic streaming potential within the osteocyte lacuno-canalicular system that activates osteocytes which, in turn, increase and maintain steady the basal current; 2) osteocytes are capable of summarizing the whole amount of energy they receive. The fact that osteocyte effect persists over time suggests the hypothesis that, under physiological loads, they have an inhibitory activity on the other cells of the osteogenic lineage and, consequently, on bone remodeling.

Discussion and functional implications

It resulted from our morphological investigations that the osteogenic cellular system (stromal cells, osteoblasts or bone lining cells, osteocytes) constitutes a functional syncytium whose variously shaped cells play different roles and have different relationships with the surrounding environment. The cytoplasmic network of stellate stromal cells is immersed in the interstitial fluid, and extends from vascular endothelium to the cells carpeting the bone surface, i.e. osteoblasts or bone lining cells. Osteocytes display an asymmetrical dendrite arborization polarized towards osteoblasts or bone lining cells, and are enclosed inside bone microcavities filled with the bone fluid compartment, having a different composition from the perivascular interstitial fluid where stromal cells are located. Osteoblasts and bone lining cells form cellular laminae in between two networks of dendrites: on their vascular side they are in contact with stromal cell processes, whereas on their bony side they are in contact with osteocyte vascular dendrites. Moreover osteoblasts and bone lining cells separate the bone fluid compartment from the perivascular interstitial fluid.

In our opinion, one of the biggest mistake made by the majority of researchers, particularly molecular biologists, was to consider the bones only in the active phases of formation and/or resorption, and thus only osteoblasts and osteoclasts were deeply studied. We should, however, bear in mind that osteoblasts and osteoclasts are transient cells; they constitute the arms *of a worker*. If we wish to detect where is the *operation center*, in order to understand how the processes of bone formation and bone resorption are first triggered and then modulated, we must focus our investigations on the events occurring in the bone cellular system starting from the resting, steady state.

According to our morphological studies, the resting phase is characterized by osteocytes, bone lining cells, and stromal cells, all connected in a functional syncytium, which extends from the bone to the

endothelial lining (Figure 1). We named this syncytium the *Bone Basic Cellular System* (BBCS) because it represents the cellular background capable of triggering and driving both processes of bone formation and bone resorption, under the control of mechanical and non-mechanical agents. It is likely that mechanical agents are first sensed by osteocytes and, in second instance, probably also by the other cells of the osteogenic lineage, whereas non-mechanical agents first affect stromal cells and then diffuse into the bone fluid volume to reach the bone lining cells and finally the osteocytes via their canalicular system. In our view BBCS represents the *bone operation center*. This view is supported by the following facts: a) bone overloading and unloading respectively induce modeling-dependent bone gain and remodeling-dependent bone loss also in adult skeleton, in which no or few osteoblasts and osteoclasts are present whereas BBCS is surely present, thus suggesting it intervenes in activating both bone formation and bone resorption; b) bone resorption was found to occur in regions less subjected to mechanical loading in biochemical osteoporoses (Lozupone and Favia, 1988; Bagi and Miller, 1994), whereas in disuse osteoporosis it takes place uniformly throughout the skeletal segments (Lozupone and Favia, 1982; Bagi and Miller, 1994), thus indicating that osteoclast activity is activated and driven by local signals which can but be issued by BBCS.

As regards osteoclasts, they are free cells that never become part of the osteogenic cell network; on the contrary, it seems likely that they should destroy stromal cells and bone lining cells, before reabsorbing the bone matrix and osteocytes. Therefore, strictly speaking, osteoclasts do not pertain to bone cells. They instead appear to be workers specialized in bone destruction and, when their activity is needed, BBCS calls them, probably by secreting osteoclast activating cytokines (RANKL), and tell them where, when and how long they have to work (Palumbo *et al.,* 2001). Osteoclasts are also under the control of blood derived systemic factors, whereas they should not be capable of sensing mechanical strains being free cells.

In conclusion, according to our view all processes of bone formation and bone resorption, occurring in response to mechanical agents and non-mechanical agents, are triggered, modulated, and stopped by the BBCS. This appears to be the real *bone operations center* capable of sensing both mechanical strains and biochemical factors and, at any moment, after having combined the two types of stimuli it issues by *wiring transmission* and/or *volume transmission* the signals that activate the processes of either bone formation or bone resorption. Such view, which ascribes a determinant function to the cells of the osteogenic lineage in the control of bone formation and bone resorption, has recently been supported by molecular biology. It has been discovered that the osteogenic cells produce the *Receptor Activator of NF-kB ligand* (RANKL) which interacts with its receptor, RANK, on hemopoietic precursors to promote osteoclast formation and activity. On the other hand the osteogenic cells also produce another protein, *Osteoprotegerin* (OPG), which bind RANKL to limit its activity and thus bone resorption (Martin, 2004; Hofbauer *et al.,* 2004).

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Cytoskeletal reorganization in skeletal muscle differentiation: from cell morphology to gene expression

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Actin cytoskeleton profoundly influence a variety of signaling events, including those related to cell growth, survival and differentiation. Recent evidence have provided insights into the mechanisms underlying the ability of cytoskeleton to regulate signal transduction cascades involved in muscle development. This review will deal with the most recent aspects of this field paying particular attention to the role played by actin dynamics in the induction of skeletal muscle-specific genes.

Key words: myogenesis, skeletal muscle, cytoskeleton, stretch-activated channels.

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Actin cytoskeleton and cell functions

The definition of cell cytoskeleton has evolved over the past half century. It includes, in fact, not only stable filamentous structures composed largely of intermediate filament proteins but also dynamic structures, such as tubulin-derived microtubular structures and actin filaments that can assemble, disassemble, and redistribute rapidly within the cells in response to signals that regulate many cellular functions, including cell shaping, intracellular organelle transport, cell motility, cell proliferation and differentiation. In particular, the understanding of the dynamics of actin-based structures may represent a major key for the comprehension of how cells respond to stimuli in the environment. Classically, filamentous actin has been considered essential for cells to form and maintain their shape. The structural basis for this event is provided by the formation of bundles of filamentous (F)-actin which are linked through focal adhesion complexes (FA) to members of the integrin family of the extracellular matrix (ECM) receptors (Geiger and Bershadsky, 2002). A large repertoire of actin-binding proteins consistently regulates the assembly and spatial organization of actin filaments (Disanza *et al*., 2005); among these are proteins that: i) promote globular (G)-actin polymerization, such as Arp2/3 complex; ii) affect depolymerization of filaments, such as the actin-depolymerizing protein ADF/cofilin and profilin at the pointed and barbed ends, respectively; iii) bind to the ends of filaments and prevent further elongation, such as tropomodulin and gelsolin); iv) crosslink actin filaments in tight bundles, namely fascin, filamin and α-actinin); vi) provide filament contraction and protein transport, such as myosin II; vii) anchor filament to membrane and to ECM receptors, including vinculin, paxillin, talin.

However, the establishment of actin cytoskeletal interaction with the extracellular matrix (cell-matrix adhesion) and with the neighboring cells (cell-cell adhesion) is important not only for the acquisition of

a peculiar cell architecture but also for the generation of forces for the remodeling of cell morphology and the promotion of the motile behavior (Disanza *et al.*, 2005; Revenu *et al.*, 2004). In fact, cell migration is a complex process which requires the dynamic turn-over of cell-substrate adhesion accompanied by *de novo* and site-directed polymerization of actin filaments at the periphery of the cells, leading to the formation of filopodia or lamellipodia.

Complexity is emerging with the observations that actin filament formation may also be critically involved in multiple cell functions, including cell cycle exit, gene expression, embryonic and tissue development, immunological response and cancer (Ingber, 2003). Actin and actin-binding proteins are, in fact, crucially involved in these processes, and also responsible for the coupling of actin-based cytoskeleton to changes in gene expression in a cell type-specific manner. Indeed, detachment of epithelial cells from the substratum leads to cell death (Frish and Francis, 1994), while fibroblasts or myoblasts respond to non-adherent conditions by reversible arrest in G0 and uncoupling of the cell cycle control from activation of muscle-specific genes (Milasincic *et al.*, 1996). This article will review some aspects of the role played by actin cytoskeletal in skeletal muscle differentiation, with the aim of summarizing the progress made in this field with particular emphasis on the molecular mechanisms linking actin remodeling to skeletal myogenic process.

Actin cytoskeleton and muscle differentiation

Activation of muscle differentiation-specific genes is controlled by the myogenic regulatory factors (MRFs), which belong to the bHLH family of transcription factors (Berkes and Tapscott, 2005; Hawke and Garry, 2001). The MRF family consists of four members: Myf5, MyoD, myogenin and MRF4, all of which bind to sequence-specific DNA elements (E-box: ...CANNTG...) present in the promoters of muscle genes. Selective and productive recognition of E-boxes on muscle promoters requires heterodimerization of MRFs with ubiquitously expressed bHLH E-proteins, rendering the formation of this functional heterodimer the key event in skeletal myogenesis. Different MRFs are expressed at different times during myogenesis. MyoD and Myf5 are required for commitment to the myogenic lineage, whereas myogenin is responsible for the induction of terminal differentiation and regulates, as a transcriptional factor, the expression of skeletal-muscle specific genes, such as actin and myosin sarcomeric proteins, muscle creatine kinase and acethylcholine receptor. MRF4 has aspects of both functions, partly subserving the specification and differentiation roles. Fusion of myoblasts into multinucleated myotubes is the terminal step of muscle differentiation. In many of these steps, cytoskeletal remodeling is required. Indeed, either disruption of actin cytoskeleton with cytochalasins or latrunculin B (Figure 1), or inhibition of SF formation with 1-butanol to block phospholipase D (PLD)-dependent SF formation, or even inhibition the acto-myosin contractility with myosin II inhibitors, have been shown to block myoblast differentiation (Formigli *et al.*, in press; Komati *et al.*, 2005; Dhawan and Helfman, 2004). Moreover, actin reorganization is required for the activation of serum response factor (SRF)-dependent muscle gene transcription (Wei *et al.* 1998; Gauthier-Rouviere *et al.*, 1996; Hill *et al.,* 1995).

A consistent body of evidence has shown that actin-mediated effects on muscle differentiation and development are dependent on the activation of members of the Rho family of small GTPase (Bryan *et al.*, 2005; Charrasse et al, 2005). In fact, the inhibition of Rho functions by pretreatment with C3 exoenzyme (a toxin isolated from *Clostridium botulinum*), or with Y-27632 (a specific Rho kinase inhibitor), or with transfection with RhoGDI (a physiological inhibitor of GTP dissociation from Rho), suppreses actin remodeling and the expression levels of myogenin, MRF4 and contractile protein genes (Komati *et al.*, 2005; Takano *et al.*, 1998; Carnac *et al.*, 1998). A number of downstream Rho-targets have indeed been identified as critical regulators of actin polymerization including, Rho kinase and mDia. Rho kinase induces SF bundling and contraction through the inhibition of myosin-light chain (MLC) kinase (Katoh *et al.*, 2001) and promotes actin polymerization through the activation of LIM kinases (LIMKs) (Sah *et al.*, 2000), while mDia1 protein modulates actin filament formation through its interaction with the actin-depolymerizing protein profilin (Watanabe *et al.*, 1997).

On the basis of the growing evidence suggesting that cell structure research may overlap with themes of gene expression and tissue development, this review will address selected aspects in this field and concentrate on the mechanisms that the authors consider novel and important for the understanding of actin-based regulation of muscle genes expres-

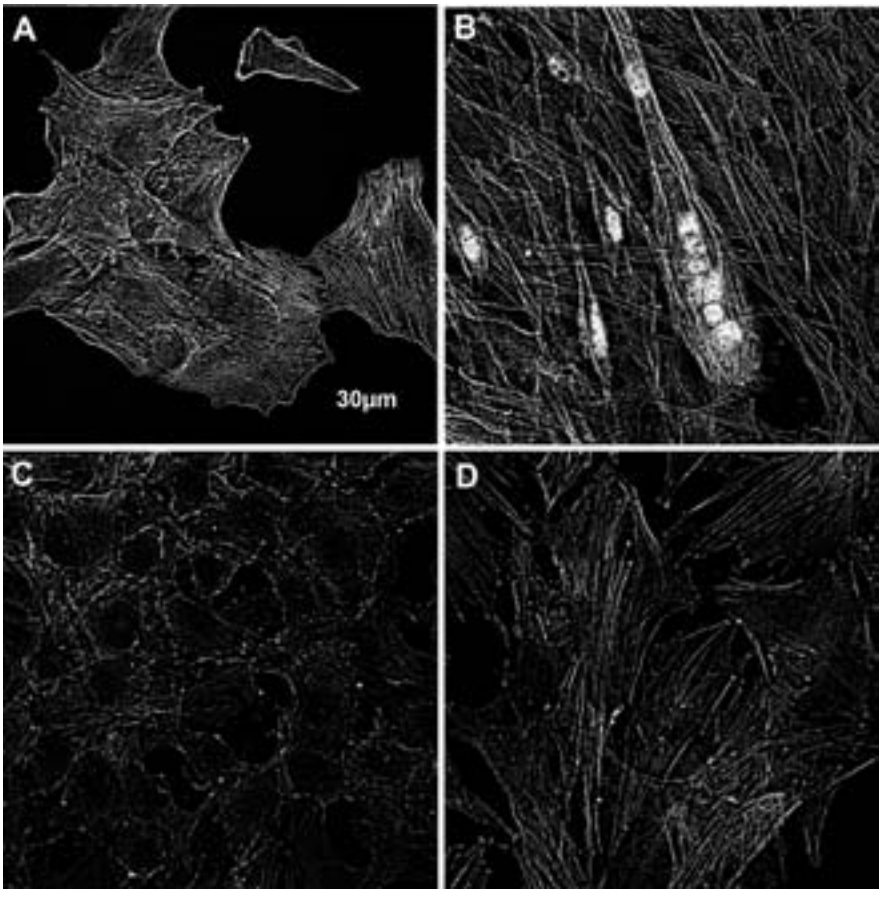

Figure 1. Effects of actin cytoskeleton on myoblast differentiation. Confocal immunofluorescence micrographs of C2C12 cells grown in DM plus S1P for 12 (A) and 72 h (B), fixed, stained for the expression of nuclear myogenin and counterstained with TRITC-conjugated phalloidin to define SF organization. Parallel experiments (C,D) have been performed in the presence of the Rho kinase inhibitor, Y-27632 to alter actin cytoskeleton. Note that the formation of myogenin-positive myotubes is strictly dependent on the integrity of the actin cytoskeleton in the early phases of myoblast differentiation.

sion. Several mechanisms linking actin cytoskeleton remodeling to cell differentiation and myogenesis will be considered: i) actin polymerization and serum response factor (SRF) activation; ii) actin polymerization and FA sites activation; iii) actin cytoskeletal interaction with gap junctional proteins, iv) actin polymerization and activation of stretch-activated channel (SACs).

Actin remodeling and SRF activation in skeletal muscle differentiation

Serum response factor is a widely expressed transcriptional factor that regulates disparate programs of gene expression linked to muscle differentiation and cellular growth, through its binding to a conserved DNA sequence, known as CarG box or serum response element (Miano, 2003).The CarG box is found in several promoters including promoters to sarcomeric-restricted genes such as skeletal alpha actin, cardiac and skeletal myosin light chain 2 (MLC-2) (Minty and Kedes, 1986). Several mechanisms exist to ensure cell-specific programs of SRF-dependent gene expression, including DNA binding, alternative splicing of SRF, chromatin remodeling of CarG boxes, and the association of SRF with a plethora of cofactors and coactivators which are cell-type specific and signal responsive. The involvement of this factor in skeletal muscle development have been clearly demonstrated by studies in which the inhibition of SRF, using antisense, dominant negative SRF mutants and neutralizing antisera, is able to suppress skeletal muscle gene expression and block myoblast-myotube transition (Wei *et al.*, 1998; Gauthier-Rouviere *et al.*, 1996; Soulez *et al.*, 1996; Vandromme M *et al.*, 1992). In addition, it has been shown that mice carrying non-functional SRF alleles do not form mesoderm and stop developing at the stage of gastrulation (Arsenian *et al.*, 1998). SRF can be activated by a huge variety of agents, including serum, lysophosphatidic acid (LPA), cytokines, tumor necrosis factor-α (TNF-α) and agents that elevate intracellular Ca^{2+} (Chai and Tarnawski, 2002). Of interest, its transcriptional activity is stimulated by changes in actin dynamics and RhoA signaling, indicating that cytoskeleton play an essential role in SRF-dependent gene expression. However, the biochemistry of SRF activation, and the signaling

pathways linking actin remodeling to SRF-dependent gene expression remain still unclear. There are several evidence that actin monomers negatively regulate SRF activation whereas actin polymerization in response to RhoA signaling stimulates SFR activity by depleting the cellular pool of inhibitory G-actin (Miralles *et al.*, 2003; Sotiropoulus *et al.* 1999). Consistent with this, the over-expression of non polymerizing β-actin mutants inhibits SRF activation (Posern *et al.*, 2002). These studies have contributed to generate the idea that G-actin could inhibit SRF directly or it could sequester cofactors required for SRF activation. Indeed, several SRF coactivators have been demonstrated to physically and functionally interact with actin (Kuwahara *et al.*, 2005), among them the muscle-specific myocardin and myocardin-related transcriptional factors (MRTFs). Upon activation of Rho signaling and actin treadmilling, these factors dissociate from actin and accumulate into the nucleus inducing SRF-dependent muscle transcription (Figure 2). Recently, a novel actin-binding protein, named striated muscle activator of Rho signaling (STARS), has been identified in early embryonic heart and skeletal muscle (Arai *et al.* 2002). This protein possesses an actin-binding domain and is associated with the I-band of sarcomere in cardiomyocytes and with stress fibers in skeletal muscle. Of interest, STARS appears to enhance actin polymerization in the presence of basal Rho activity and stimulates the transcriptional activity of SRF by inducing the nuclear accumulation of MRTF-A and B (Kuwahara *et al.*, 2005). Thus a model has been proposed wherein Rho activates STARS, which upon binding to actin, promotes actin polymerization. This event releases MRTFs from the inhibitory influence of G-actin, allowing their nuclear import and the stimulation of SRF activity and, eventually, myogenesis.

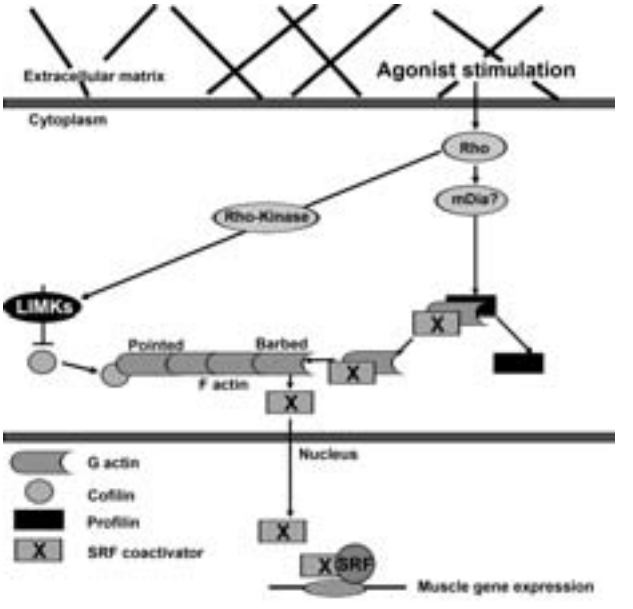

Figure 2. Model for SRF activation via actin reorganization. Agonist stimulation activates Rho and Rho kinase-dependent actin polymerization. Rho kinase activates LIM kinases (LIMKs) which, by phosphorylation of actin-depolymerizing cofilin, inhibit its action and stabilize actin filament at the pointed ends. Rho activates mDia which, by inhibition of the actin-depolymerizing protein profilin, enhances actin polymerization at the barbed ends. Upon binding to the barbed ends, G-actin may release SRF-coactivators ("X"), which, in turn, migrate into the nucleus and stimulate SRF-dependent muscle-gene expression.

Actin polymerization and FA site activation in skeletal muscle differentiation

Previous investigations have shown that organization of SF in response to receptor stimulation provide the scaffolds for the assembly of FA and the basis for cell-matrix interaction (Burridge *et al.*, 1997). These events are mainly mediated by Rho activation and by its effector, Rho kinase, which enhancing myosin II light chain (MLC) phosphorylation, both by inactivation of MLC phosphatase or direct phosphorylation of MLC, stimulates actin and myosin interaction and, in turn, actin filaments bundling and FA protein clustering (Charnowska and Burridge, 1996). However, other signaling events driven from the outside of the cells, namely from integrin-mediated-cell adhesion are required to form FA complexes (Cary *et al.*, 1999). Indeed, the binding of integrins with molecules of extracellular matrix (fibronectin, laminin and collagen) leads to their clustering and activation of a series of intracellular events culminating in the reorganization of actin cytoskeleton at the sites of engagement and in the recruitment of FA proteins (Juliano, 2002; Turner, 2000). The coupling between integrin and more *conventional* signaling receptors allows cells to integrate positional information concerning cell matrix contact with information about the availability of growth or differentiation factors (Figure 3). This is particularly true in consideration that FA sites are more than just structural sites linking cytoskeleton to ECM, and are regions of important signal transduction cascades involved in numerous cell functions, including cell differentiation and skeletal muscle formation (Wozniak *et al.*, 2004; Goel and Dey, 2002). In fact, these sites contain sev-

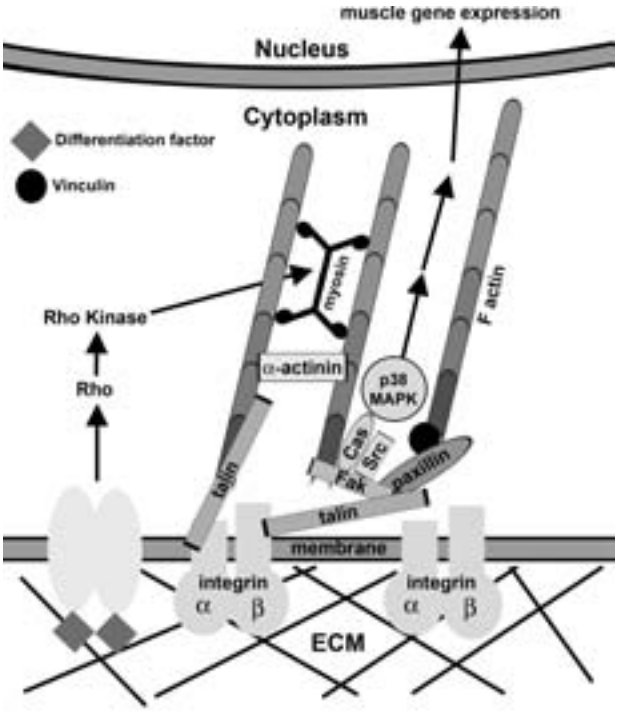

Figure 3. Model for FA activation. Integrin activation after engagement with ECM or induction of Rho signaling in response to receptor activation lead to actin cytoskeletal reorganization and to accumulation of FA proteins at the sites of engagement. Subsequently, FAK becomes phosphorylated thus creating the binding sites for adaptor proteins (paxillin and Cas) and for Src. Phosphorylation of Src by FAK triggers MAPK cascade, thereby resulting in gene expression.

eral tyrosine kinases and adaptor proteins, such as paxillin and p130Cas, which, acting as signaling scaffolds for the components of FA, allow them to properly interact with their substrate. FAK, a non receptor tyrosine kinase, has emerged as a key signaling component of FA. It is activated by autophosphorylation that is initiated by its clustering into FA sites. When phosphorylated, FAK creates docking sites for the binding of SH2-containing proteins and regulates activation of additional kinases and phosphatases, acting as a switch for multiple signaling outputs (Parsons, 2003; Oktay *et al.*, 1999). Of interest, FAK phosphorylation has been associated with the induction of skeletal myogenesis (Huang *et al.*, 2006; Clemente *et al.* 2005; Wozniak *et al.*, 2004; Goel and Dey, 2002; Lee *et al.*, 1999), namely through the activation of members of Src protein family, of mitogen-activated protein kinase (MAPK) family (namely p38MAPK) and of phosphatidylinositol (PI)3-kinases, whose involvement in Rho-dependent muscle differentiation has been well established (Khurana and Dey, 2003; Cabane *et al.*, 2003; Goel and Dey, 2002; Aikawa *et al.*, 2002; Wei *et al.*, 2001). It is worthy to point out that FAK phosphorylation and activation critically depends on the integrity of actin cytoskeleton during muscle cell differentiation (Goel and Dey, 2002; Lee *et al.*,1999), thus supporting a model in which cytoskeletal remodeling may trigger internal signaling and be converted into changes of gene expression (Wozniak *et al.*, 2004).

Actin remodeling and gap junctional proteins in skeletal muscle differentiation

A consistent body of evidence has demonstrated that specific types of cell contacts, the gap junction (GJ) are present between skeletal muscle cells. GJ are composed of intercellular channels formed by the conjunction of two hemichannels made of six proteins belonging to the connexin (Cx) family, whose Cx43 is the most widely expressed member (Saez *et al.*, 2003). Thus far, these structures have not been found between mature innervated muscle fibers and exist as transitory state during myoblast differentiation. It has long been suggested that the transfer of small metabolites and signaling molecules between adjacent skeletal muscle cells through the gap junctions, plays a fundamental role in the regulation and coordination of myoblast differentiation (Constantin *et al.*, 2000). Indeed, the application of intercellular communication inhibitors (Proulx *et al.*, 1997) and the inducible deletion of Cx43 proteins (Araya *et al.* 2005, 2003) dramatically affect myogenesis. Notably, our recent findings provide novel evidence for a role of actin cytoskeleton in the Cx43-mediated effects on myogenesis (Squecco *et al.*, 2006). In particular, the reduced interaction between a mutated form of Cx43 and actin and cortactin, as well as the inhibition of p38 MAPK-dependent signaling pathway essential for this interaction, are able to completely inhibit the expression of myogenic marker proteins (myogenin, myosin heavy chain, caveolin-3) and the achievement of the fully differentiated phenotype elicited by sphingosine 1-phosphate, a bioactive lipid that participates in the regulation of myoblast biology (Squecco *et al.*, 2006; Formigli *et al.*, 2005; Donati *et al.*, 2005; Formigli *et al.*, 2004; Meacci *et al.*, 2003; Meacci *et al.*, 2002; Formigli *et al.*, 2002). Notably, the drastic inhibition of myogenesis occurred even if the intercellular conductance was only partially affected in these conditions. These data have led to the suggestion that Cx43 expression may also stimulate skeletal myogenesis through

gap-junction independent mechanisms. The finding concerning the role of Cx43 as membrane-cytoskeleton anchor protein in myoblasts may indeed represent a crucial aspect in the molecular mechanisms involved in the promotion of muscle gene expression by Cx43 expression. These data are in agreement with recent studies that pointed out the important role of GJ-independent functions of Cx43 in the regulation of many cellular processes such as growth, survival and migration (Jiang and Gu, 2005; Giepmans, 2004; Stout 2004; Dang *et al*. 2003; Morby *et al*., 2001; Omori and Yamasaki, 1998; Huang *et al*., 1998). Moreover, accumulating evidence has demonstrated a direct interaction of Cx43 C-terminus with cytoskeletal proteins, such as the tight junction protein Zona Occludens-1 (ZO-1) (Sing *et al*., 2005; Tokyofoku, 2001), tubulin (Giepmans *et al*., 2001) and the actin binding protein drebrin (Butkevich *et al*., 2004) as well as with signal molecules such as c-src and v-src tyrosine kinase (Giepmans *et al*., 2001).

Actin polymerization and SAC-activation in skeletal muscle differentiation

Stretch-activated cation channels have been described in a huge variety of cells in different organisms ranging from bacteria to mammals. These channels allow the passage of cations, like Na^+, K^+, $Mg2^+$, and Ca^{2+} (Munevar *et al*., 2004) and participate in several physiological processes, ranging from cell volume regulation and muscle contraction to cell differentiation (Jakkaraju *et al.* 2003; Minke and Cook, 2002). In particular, recent reports have demonstrated that SACs activate second messengers, namely Ca^{2+} and Ca^{2+}-dependent signal pathways, necessary for modulating gene expression in different mammalian cells (Kumar *et al*., 2003; Inoh *et al*., 2002). Abnormal regulation of SACs and the excessive increase in the intracellular Ca^{2+} concentration also contribute to the pathogenesis of several diseases, including muscular dystrophy and cardiac arrhythmias (Kumar *et al*., 2004). The mechanical distension of the plasma membrane modulates the ion-transporting activity of these channels by producing conformational changes that alter their opening or closing rates through the distortion of the associated lipid layer or through the displacement of intramolecular gating domains. In such a view, activation of SACs represents an important transduction mechanism that convert mechanical forces into electrical and biochemical signals in physiological process (Ingber, 2006). Single molecule force spectroscopy studies have shown that individual peptide domains within proteins found in the actin cytoskeleton and FA complex unfold when SACs are mechanically extended, suggesting a close morphological and structural interaction between these channels and cytoskeletal elements (Oberhauser *et al*., 1999; Janmey, 1998). However, the functional impact of actin cytoskeleton reorganization on SACs activity remains controversial and seems to be strictly dependent on the different status of microfilaments in specialized cells. Previous studies have documented that actin cytoskeletal disruption with cytochalasins or latrunculin increases the channels' sensitivity to stretch and promotes SAC activation in cultured fibroblasts (Wu *et al*., 1999). On the other hand, actin cytoskeletal disassembly causes a decrease in single current and conductance of SACs in myeloid leukemia cells (Staruschenko *et al*., 2005), suggesting that the organization of the cortical microfilaments may be determinant in negatively modulate channel function in these cells. In addition, recent reports from our group have demonstrated that not only actin depolymerization but also actin polymerization and SF formation may modulate SAC function in myoblastic cells (Formigli *et al*., 2005). Using an atomic force microscopy, we have also shown that the formation of a well structured actin cytoskeleton is indeed capable to impose a mechanical strain on the myoblast plasma membrane and lead to SAC-mediated Ca^{2+} current inwards (Paternostro *et al*., 2006). Notably, we have also observed that SF formation and SAC activation during the early phases of myoblast differentiation play a pivotal role in the regulation of skeletal myogenesis (Formigli *et al*., in press). Indeed, consistent with a previous investigation (Wedhas *et al*., 2005), the treatment of C2C12 myoblasts with Gadolinium chloride, a specific SAC channel blocker, inhibits myotube formation and the expression of myogenic markers of differentiation. These effects are modulated by cytoskeletal components and are abolished after treatment with actin disrupting agents, in perfect agreement with a model whereby actin polymerization modulates SAC opening and Ca^{2+} channel inward current and, in turn, the activation of Ca^{2+}-mediated pathways leading to muscle-specific gene expression.

Concluding remarks

The dominant view in cell biology is that cell function is controlled by soluble factors and adhesive ligands, which exert their effects by binding to cell surface receptors, thereby activating signal transduction cascades inside the cell, leading to modifications in gene expression. Complexity is now emerging from the growing evidence suggesting that changes in actin organization may represent a critical step in the cell response to stimuli, linking receptor activation with the generation of regulatory signals. In particular, several specific signaling involved in skeletal muscle differentiation, such as SRF, paxillin and FAK, Cx43-formed channel and SACs activation can be considered as downstream effectors of actin cytoskeleton and its dynamic state. The analyses of the relationship existing between actin dynamics and muscle development will certainly shed light on the understanding of the mechanisms underlying satellite cell activation and differentiation during skeletal muscle regeneration and also on the identification of new therapeutic strategies in muscle diseases, such as dystrophy, characterized by alterations in cytoskeletal organization and cell adhesion.

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Sarcoglycan subcomplex in normal and pathological human muscle fibers

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Sarcoglycans are a sub-complex of transmembrane proteins which are part of the dystrophin-glycoprotein complex (DGC). They are expressed above all in the skeletal, cardiac and smooth muscle. Although numerous studies have been conducted on the sarcoglycan sub-complex in skeletal and cardiac muscle, the manner of distribution and localization of these proteins along the non-junctional sarcolemma is still not clear. Furthermore, there are unclear data about the actual role of sarcoglycans in human skeletal muscle affected by sarcoglycanopathies. In our studies on human skeletal muscle, normal and pathological, we determined the localization, distribution and interaction of these glycoproteins. Our results, on normal human skeletal muscle, showed that the sarcoglycans can be localized both in the region of the sarcolemma over the I band and over the A band, hypothesizing a correlation between regions of the sarcolemma occupied by costameres and the metabolic type of the fibers (slow and fast). Our data on skeletal muscle affected by sarcoglycanopathy confirmed the hypothesis of a bidirectional signaling between sarcoglycans and integrins and the interaction of filamin2 with both sarcoglycans and integrins. In addition, we have recently demonstrated, in smooth muscle, the presence of α-SG, in contrast with data of other Authors. Finally, we analyzed the association between contractile activity and quantitative correlation between α- and ε-SG, in order to better define the arrangement of sarcoglycan subcomplex.

Key words: sarcoglycans, skeletal muscle, cardiac muscle, human, sarcoglycanopathy, muscular diseases.

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The sarcoglycan subcomplex (SGC) is a well-known system of interaction between extracellular matrix and sarcolemma-associated cytoskeleton in skeletal and cardiac muscle. This subcomplex is made up of a series of transmembrane proteins (Ettinger *et al.*, 1997) which, together with other components of the dystrophin-glycoprotein complex (DGC), regulate interaction between the cytoskeleton and extracellular matrix in skeletal muscle and cardiac muscle. In this way, these glycoproteins stabilize the sarcolemma of the myofibrils and the cardiomyocytes and protect the muscle fibers from any possible damage provoked by continuing cycles of contraction and relaxation (Ervasti *et al.*, 1990).

The SGC is made up of four glycoproteins linked, by a lateral binding, to β-dystroglycan, (Crosbie *et al.*, 1997), α-sarcoglycan, 50 kD, a type I protein, that is with the NH-terminal on the intracellular side, the β-, γ- and the δ-sarcoglycans, 43 kD, 35 kD and 35 kD respectively, all type II proteins, that is with the NH-terminal on the extracellular side (Yoshida *et al.*, 1994). The α-sarcoglycan and the γ-sarcoglycan are expressed only in muscular tissue, while the other sarcoglycans have a wider distribution.

A widely expressed fifth sarcoglycan with significant homology to α-sarcoglycan, ε-sarcoglycan, has been identified; this sarcoglycan is expressed in both muscle and non-muscle cells, and in embryos as well as adults (Ettinger *et al.*, 1997). It is hypothesized that ε-sarcoglycan might replace α-sarcoglycan in smooth muscle, forming a novel sarcoglycan subcomplex consisting of ε-, β-, γ-, and δ-sarcoglycan (Barresi *et al.*, 2000). Thus, it is possible that sarcoglycans, like other components of the DGC, may play a key role for embryonic development and for viability of non-muscle tissues (Ettinger *et al.*, 1997).

Recently, a novel mammalian sarcoglycan, ζ-sarcoglycan, highly related to γ-sarcoglycan and δ-sar-

coglycan, has been identified (Wheeler *et al.*, 2002). This protein is encoded by a gene on human chromosome 8. By using a ζ-sarcoglycan-specific antibody, it has been demonstrated that ζ-sarcoglycan was expressed in muscle and co-immunoprecipitated with other sarcoglycan components. Moreover it has been hypothesized that ζ-sarcoglycan may be a candidate gene for muscular dystrophy and a possible mediator of muscle membrane instability in DGC-mediated muscular dystrophy (Wheeler *et al.*, 2002).

On this basis, growing evidence suggest that there are two types of sarcoglycan complexes; one, in skeletal and cardiac muscle, consisting of α-, β-, γ- and δ-sarcoglycan, and the other, in smooth muscle, containing β-, δ-, ζ- and ε-sarcoglycan (Wheeler *et al.*, 2002). ε-sarcoglycan may substitute for α-sarcoglycan in a subset of striated muscle complexes. Our recent study on smooth muscle fibers, hypothesized an exameric structure of SGC (Anastasi *et al.*, 2005).

The sarcoglycans play a key role in the pathogenesis of many muscular dystrophies, such as Duchenne and Becker muscular dystrophies and sarcoglycanopathies (Bönnemann *et al.*, 2002). In fact, recent developments in molecular genetics have demonstrated that mutation in each single sarcoglycan gene, respectively 17q, 4q, 13q and 5q, causes a series of recessive autosomal dystrophin-positive muscular dystrophies, not accompanied by a lack of dystrophin, called sarcoglycanopathies or Limb Girdle Muscular dystrophies (LGMD type 2D, 2E, 2C and 2F) (Roberds *et al.*, 1994).

It has recently been shown that the assembly of the SGC begins from a core condition of stability made up, at first, of β-sarcoglycan and δ-sarcoglycan. Later α- and γ-sarcoglycans are also involved which activate the maturation phase of the complex; finally, dystrophin, which plays a mechanical role in the activation of links in the context of the SGC (Hack *et al.*, 2000) is also assembled. Based on this, the absence of damage induced by contraction in γ-sarcoglycan deficient muscles, would suggest a non-mechanical role for this sarcoglycan, or the sarcoglycan complex, in skeletal muscle fibers. Some authors have recently hypothesized that the absence of one or all of the sarcoglycans, independently or in the presence of dystrophin, leads to an alteration in the permeability of the cellular membrane and to apoptosis (Hack *et al.* 2000).

On this ground, it is demonstrated that the sarcoglycans are separated into two subunits: one consisting of α-sarcoglycan and the other consisting of β-, γ- and δ-sarcoglycan (Anastasi *et al.*, 2003a, Anastasi *et al.*, 2003b; Anastasi *et al.*, 2004) in which the association between β- and δ-sarcoglycan is particularly strong. The tight association between β- and δ-sarcoglycan confirms the hypothesis that they may constitute a functional core for the assembly of the sarcoglycan subcomplex (Hack *et al.*, 2000).

This tight link suggests that β- and δ-sarcoglycan may be the functional core for the assembly of the sarcoglycan sub-complex. The presence of γ- and α-sarcoglycan is required, in a successive stage, to allow the right assembly and processing of the subcomplex; finally, dystrophin is also assembled. (Bönnemann *et al.*, 1995). Mutations in either β- or δ-sarcoglycan are expected to have an important effect on the sarcoglycan sub-complex, determining the absence or the reduction of all sarcoglycans in the sarcolemma. Mutations of α-sarcoglycan cause only minor changes in the sarcoglycan sub-complex, suggesting that its association with the other sarcoglycans is weak and that the protein is spatially separated from other glycoproteins. (Yoshida *et al.*, 1994; Barresi *et al.*, 1997; Chan *et al.*, 1998).

Moreover, it has been hypothesized (Yoshida *et al.*, 1998) a bidirectional signaling between sarcoglycans and integrins. The integrins are a family of transmembrane heterodimeric receptors that play a key role in the process of cell adhesion, linking the extracellular matrix to the actin cytoskeleton and providing bidirectional transmission of signals between the extracellular matrix and the cytoplasm. The integrin receptor family includes at least 14 distinct α subunits and 8 β subunits. It is well known that α7B and β1D integrins predominate in the adult skeletal and cardiac muscle. The presence of vinculin, talin and integrins at a costameric level suggests that costameres may be considered as an adherens junction-like system between cell and extracellular matrix.

We showed, performing a immunofluorescence study, a colocalization between sarcoglycans and integrins. On this basis, these data demonstrated, according to hypothesis of Yoshida *et al.* (1998), the existence of a bidirectional signalling between sarcoglycan and integrin (Anastasi et al., 2003b; Anastasi *et al.,* 2004). This is in agreement with the reported presence of filamin2 (FLN2) as interactor with both sarcoglycans and integrins.

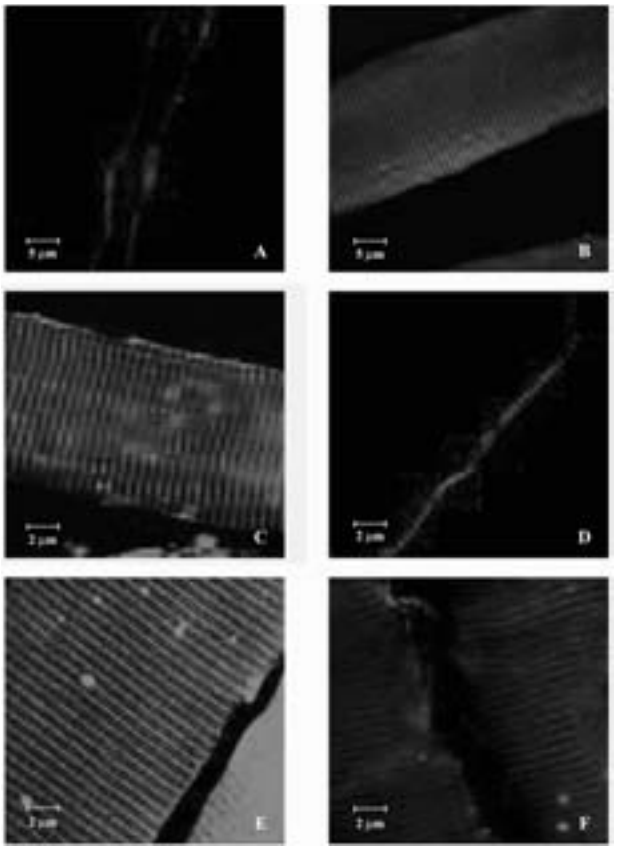

Figure 1. Longitudinal sections of human skeletal muscle affected by LGMD2D and LGMD2C immunolabeled with sarcoglycan antibodies. In LGMD2D α-sarcoglycan staining appeared severely reduced (A), other tested proteins staining were clearly detectable; in C we showed γ-sarcoglycan (C). In LGMD2C, α-sarcoglycan (B), staining showed a normal pattern; γ-sarcoglycan (D) staining appeared severely reduced. The analysis of filamin2 revealed that in LGMD2D filamin2 staining appeared clearly detectable (E), while in LGMD2C appeared severely reduced (F).

The FLN2 membrane increase in LGMD patients suggests that this protein is binding other membrane bound proteins other than the sarcoglycans. A logical candidate for this second interacting protein would be β1 integrin given that both of the other filamin family members bind to this subunit in other cells.

Most reports about filamin functions include a role in actin polymerization, a critical process for the regulation of the contractile apparatus in skeletal muscle as well as cell structure, in the organization of membrane receptors with signalling molecules and in mechanoprotection in other tissues. These processes can regulate cell behavior by providing the cell with the information necessary for making decision regarding cell shape, adhesion and migration, growth and differentiation, apoptosis and survival.

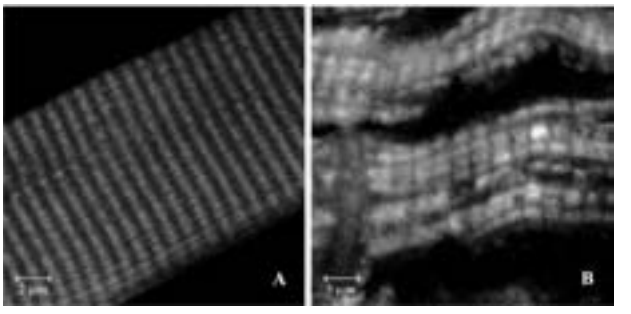

Figure 2. Longitudinal sections of human skeletal muscle (A) and human cardiac muscle (B) immunolabeled with α- and γ-sarcoglycan antibodies. All sarcoglycans appear as costameric bands at regular intervals.

Our recent studies, carried out on human skeletal muscle by subjects affected by α-, and γ-sarcoglycanopathy, showed that filamin2 staining pattern is almost absent in γ-sarcoglycanopathy, in which also the subunit β-γ-δ- staining is absent, while this protein has normal staining pattern in α-sarcoglycanopathy, in which also the subunit β-γ-δ- has normal values of fluorescence (Anastasi *et al.*, 2005). These data are summarized in the Figure 1, in which we showed the sarcoglycan staining patterns in α-sarcoglycanopathy (LGMD2D) and γ-sarcoglycanopathy (LGMD2C). In LGMD2D, α-sarcoglycan staining was almost absent (Figure 1a). The analysis of other sarcoglycans showed a normal staining pattern; in Figure 1c we showed only γ-sarcoglycan staining is shown. In LGMD2C, α-sarcoglycan fluorescence had a normal pattern (Figure 1b), while immunofluorescence of other sarcoglycans appeared severely reduced, in Figure 2d only γ-sarcoglycan staining is shown.

Filamin2 staining pattern was normal in LGMD2D (Figure 1e), and severely reduced in LGMD2C (Figure 1f).

These data showed that the behaviour of this protein could be due to the lack of both γ-sarcoglycan and β1D-integrin in γ-sarcoglycanopathy, with consequent lack of interaction with FLN2 and its following disappearance from sarcolemma. These results seems to support the Thompson' hypothesis (1998) about the role of β1 integrin as a second interacting protein with filamin2.

The SGC is included in the dytrophin-glycoprotein complex (DGC) made up of sarcoplasmic subcomplex and a dystroglycan subcomplex. The sarcoplasmic subcomplex is made up of the dystrophin, dystrobrevin and syntrophins. The dystroglycan subcomplex is made up of α- and β-dystroglycan, both

Table 1. The first part of table summarizes the results of double localization reactions carried out to verify colocalization of each sarcoglycan with each other proteins (sarcoglycans, dystrophin, β-dystroglycan, and vinculin-talin-integrin system proteins). These data show that the sarcoglycans colocalize among themselves in different percentages. In the second part of table, are reported the percentages of colocalization and no colocalization of sarcoglycans with actin, in order to examine the localization of the proteins.

Reaction	*Colocalization (%)*	*Partial localization (%)*	*No Colocalization (%)*
α-SG / β-SG	94	0	6
α-SG / γ-SG	95	0	5
α-SG / δ-SG	93	0	7
α-SG / β-DG	90	8	2
α-SG / Dystrophin	89	11	0
β-SG / γ-SG	100	0	0
β-SG / δ-SG	100	0	0
β-SG / Dystrophin	93	7	0
γ-SG / Dystrophin	92	8	0
δ-SG / β-DG	91	9	0
δ-SG / Dystrophin	90	10	0
α7β / α–SG	93	0	7
α7β / β–SG	100	0	0
β1D / α-SG	94	0	6
β1D / β-SG	100	0	0
Vinculin / α-SG	94	0	6
Vinculin / β-SG	100	0	0
Talin / α-SG	95	0	5
Talin / β-SG	100	0	0

Reaction	I band *(%)*	A band *(%)*
α-SG / Actin	26	74
β-SG / Actin	33	67
γ-SG / Actin	27	73
δ-SG / Actin	30	70
α7B / Actin	32	68
β1D / Actin	33	67

essentials in cell surface matrix organization. There are conflicting data about the localization and distribution of SGC, and his colocalization with other components of DGC and the vinculin-talin-integrin system. Some Authors demonstrated that these proteins are localized in the region corresponding to the I band of the underlying sarcolemma (Pardo *et al.*, 1983), while other Authors believe that these proteins are localized, together dystrophin and vinculin, in the sarcolemma above the A band (Minetti *et al.*, 1992).

Our studies, carried out on normal human skeletal and cardiac muscle showed that all sarcoglycan have a costameric distribution, confirming the previous hypothesis (Mondello *et al.*, 1996) of costameres as the *machine protein*. The costameric distribution is showed in Figure 1 by single localization, using a stack of 16 sections of 0.8 μm of scan steps, carried out on 20 μm thick cryosections of skeletal muscle, on which indirect immunofluorescence reaction had been performed using anti-α-sarcoglycan (Figure 2a) and anti-γ-sarcoglycan (Figure 2b) antibodies in single localizations.

Sarcoglycans colocalize, in different percentages, with other proteins (sarcoglycans, dystrophin, β-dystroglycan, and vinculin-talin-integrin system proteins) and all are localized, in different percentages, both in the regions of the sarcolemma over I band and in the regions of the sarcolemma over A band.

It is known that skeletal muscle is made up of both slow and fast fibers in different proportion

(Johnson *et al.*, 1973), while cardiac muscle is made up exclusively of slow fibers with a highly oxidative metabolism. Thus, we hypothesized that slow fibers are characterized by localization of costameric proteins on the region of the sarcolemma over band I, while fast fibers by localization of the same proteins in the region over band A (Anastasi *et al.*, 2003a, Anastasi *et al.*, 2003b). Moreover, these data confirm the hypothesis of two subunit, one consisting of a-sarcoglcan and other formed by β–γ–δ–sarcoglycan (Anastasi *et al.*, 2003a; Anasatsi *et al.*, 2004). All these data are summarized in Table 1.

It will be intriguing, besides, to integrate these studies with molecular biology techniques; in fact the definition of patterns in immunohistochemical profile would be important to guide the genetic analysis directly to the responsible gene and abbreviate molecular genetic investigations (Bönnemann *et al.*, 2002).

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Stem cell-mediated muscle regeneration and repair in aging and neuromuscular diseases

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One of the most exciting aspirations of current medical science is the regeneration of damaged body parts. The capacity of adult tissues to regenerate in response to injury stimuli represents an important homeostatic process that until recently was thought to be limited in mammals to tissues with high turnover such as blood and skin.
However, it is now generally accepted that each tissue type, even those considered post-mitotic, such as nerve or muscle, contains a reserve of undifferentiated progenitor cells, loosely termed stem cells, participating in tissue regeneration and repair.
Skeletal muscle regeneration is a coordinate process in which several factors are sequentially activated to maintain and preserve muscle structure and function upon injury stimuli. In this review, we will discuss the role of stem cells in muscle regeneration and repair and the critical role of specific factors, such as IGF-1, vasopressin and TNF-α, in the modulation of the myogenic program and in the regulation of muscle regeneration and homeostasis.

Key words: IGF-1, vasopressin, oxytocin, TNF-α, satellite cell.

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Stem cells and muscle regeneration

The contribution of satellite cells to muscle regeneration

Regeneration of adult skeletal muscle is a highly coordinated program that partially recapitulates the embryonic developmental program. The major role in growth, remodeling and regeneration is played by satellite cells, a quiescent population of myogenic cells residing between the basal lamina and the plasmalemma (Mauro, 1961) and rapidly activated in response to appropriate stimuli. RT-PCR analysis and gene targeting strategies (Cornelison *et al.,* 1997; Charge *et al.,* 2004) revealed that satellite cells present a heterogeneous profile of gene expression depending on the functional stage of the myogenic program. Once activated, satellite cells express factors involved in the specification of the myogenic program such as Pax-7, desmin, MNFα, Myf-5 and MyoD. Activated satellite cells proliferate as indicated by the expression of factors involved in cell cycle progression such as PCNA and by the incorporation of BrDU. Ultimately the committed satellite cells fuse together or to the existing fibers to form new muscle fibers during regeneration and muscle repair (Charge *et al.,* 2004). This aspect of muscle regeneration is hampered in several muscle diseases, including aging and muscular dystrophies.

In this context, myoblast cell therapy has therefore been extensively explored as a promising alternative to correct genetic diseases by contributing to tissue regeneration. Replacement of diseased muscles with healthy and functional muscle fibers has long been a major therapeutic strategy for muscular dystrophies (Grounds, 2000). However, the failure of injected committed cells to survive in the recipient animals and successfully engraft within their target organs has proven disappointing. Indeed, even under optimized environment for myoblast transplantation, such as in an immunodeficient, irra-

diated mdx host, the majority of transplanted cells underwent rapid death (Beauchamp *et al.,* 1999; Smythe *et al.,* 2001). Therefore, the poor survival of injected cells (less than 1%), minimal migration from injection site (1 mm) and rapid senescence of the surviving population, has failed to produce satisfactory protocols of muscle regeneration that might be considered for therapeutic purposes.

Several lines of research have been employed to increase the survival of injected myoblasts. Modulation of the inflammatory reaction to foreign cells is emerging as a necessary prerequisite for effective clinical applications of myoblast transplantation (Guerette *et al.,* 1997; Hodgetts *et al.,* 2000; Hodgetts *et al.,* 2003). Thus, integrating gene and cell therapy approaches may circumvent the major problems associated with the survival of transplanted cells, enhancing cell engraftment and improving muscle regeneration. The alternative approach is represented by skeletal muscle tissue engineering *in vitro* (recently reviewed by Bach *et al.* (Bach *et al.,* 2004). The latter aims to use *in vitro*-designed and pre-fabricated artificial muscle tissue equivalent to be implanted after differentiation has taken place. This approach, though, while very intriguing (Bach *et al.,* 2006) is still far from being suitable for clinical practice, differently from other tissue reconstructions. In summary, these studies emphasize how the restorative potential of pathological muscle is dependent not only on the presence of satellite cells, but also on the support of optimal environmental cues.

This hypothesis is supported by recent experimental evidences. It has been suggested that the decline in the regenerative potential of senescent muscle is mainly due to a decline in satellite cell number (Schultz *et al.,* 1982). However, other evidences suggested alternative explanations. Conboy reported that the dramatic age-related decline in myoblast generation in response to injury is due to an impairment of activation rather than a decline in number of satellite cells, (Conboy *et al.,* 2003) demonstrating that Notch signaling plays a pivotal role in satellite cell activation and cell fate determination. Indeed, to examine the influence of systemic factors on aged progenitor muscle cells, this group recently established parabiotic pairings (that is, a shared circulatory system) between young and old mice (heterochronic parabiosis), exposing old mice to factors present in young serum (Conboy *et al.,* 2005). Notably, heterochronic parabiosis restored the activation of Notch signaling as well as the proliferation and regenerative capacity of aged satellite cells.

The limitation of senescent skeletal muscle to sustain an efficient regenerative mechanism raises a question as to whether this is due to the intrinsic ageing of stem cells or rather to the impairment of stem-cell function in the aged tissue environment.

The contribution of stem cells to muscle regeneration

The discovery of stem cell lineages in many adult tissues has challenged the classic concept that stem cells in the adult are present in only a few locations, such as the skin or bone marrow, and are committed to differentiate into the tissue in which they reside. In addition, several evidences suggested that the migration of circulating stem cells into the injured area represents the mechanisms by which different tissues are repaired (Blau *et al.,* 2001).

Searches for adult stem cells have relied on information derived primarily from studies of stem cells in the bone marrow, which must renew themselves daily to maintain the body's blood supply. An understanding of the plasticity of adult stem cells initially grew from observations that donor cells were found in non-hematopoietic tissues in the recipients of bone marrow transplants. Indeed, accounts of the repopulation of adult organs by bone marrow-derived stem cells suggest that under the right conditions, they can contribute to virtually any part of the body. However, this phenomenon seems a rare event and presents limitations for an efficient tissue repair. It has been proposed that adult bone marrow-derived cells contribute to muscle tissue in a step-wise biological progression (LaBarge *et al.,* 2002). Following irradiation-induced damage, transplanted bone marrow-derived cells become satellite cell; alternatively, they may fuse directly into regenerating muscle fibers (Camargo *et al.,* 2003). However, in all animal studies to date, it has been necessary to replace host bone marrow with marked progenitor cells to prove their provenance. This experimental manipulation inevitably involves lethal irradiation of the host animal, a process that is emerging as a necessary prerequisite for bone marrow engraftment into injured muscle (Morgan *et al.,* 2002). In any case, the total number of bone marrow stem cells recruited to a muscle fate in these studies appears still insufficient to be of therapeutic benefit. In fact it has been reported that the

poor recruitment of haematopoietic stem cells into the dystrophic muscle of the mdx mouse is the major obstacle for muscle regeneration and therefore for the rescue of the genetic disease (Ferrari *et al.,* 2001).

A new class of vessel associated fetal stem cells, termed mesoangioblasts, has been isolated (Cossu *et al.,* 2003). These cells show profiles of gene expression similar to that reported for hematopoietic, neural, and embryonic stem cells. Mesoangioblasts can differentiate into most mesoderm (but not other germ layer) cell types when exposed to certain cytokines or to differentiating cells (Cossu and Bianco, 2003). Intra-arterial mesoangioblast delivery was effective in restoring expression of α-sarcoglycan protein and of the other members of the dystrophin glycoprotein complex in treated α-sarcoglycan null mice (Sampaolesi *et al.,* 2003). Restoration of sarcoglycan expression was also associated with a marked reduction of the fibrosis and complete functional recovery of treated muscle. More recently, the same group demonstrated that mesoangioblast stem cells ameliorate muscle function in dystrophic dogs, qualifying mesoangioblasts as candidates for future stem cell therapy for Duchenne patients (Sampaolesi *et al.,* 2006).

Although stem cells offer a new tool for regeneration in muscle disease, the signalling and molecular pathways involved in recruitment and myogenic commitment of progenitors cells is an important question that remains to be satisfactorily addressed. In addition, the environment in which these stem cells operate represents another important determinant for cell survival and differentiation, which may be compromised in the dystrophic milieu.

The regenerative capacity of skeletal muscle is influenced by several factors (Charge *et al.,* 2004), including growth factors and hormones, secreted in an autocrine/paracrine manner. Alterations in these parameters compromise the ability of skeletal muscle to sustain a regenerative process, leading to repeated episodes of incomplete muscle repair and therefore to muscle wasting.

The importance of the tissue niche: the critical role of IGF-1

One of the crucial parameters of tissue regeneration is the microenvironment in which the stem cell population should operate. Stem cell microenvironment, or niche, provides essential cues that regulates stem cell proliferation and that directs cell fate decisions and survival. Moreover, loss of control over these cell fate decisions might lead to cellular transformation and cancer.

Studies on stem cell niche leaded to the identification of critical players and physiological conditions that improve tissue regeneration and repair. Among growth factors, IGF-1 exerts anabolic effects in different tissues, including skeletal muscle where it plays a key role in growth, hypertrophy and muscle regeneration (Musarò *et al.,* 2006).

In the last decade we studied the molecular and cellular mechanisms underlying muscle hypertrophy and regeneration in skeletal muscle. We generated transgenic mice in which the local isoform of IGF-1 (mIGF-1) is driven by MLC promoter (MLC/mIGF-1) (Musarò *et al.,* 2001). Under the control of skeletal muscle-restricted, postmitotic regulatory elements, the MLC/mIGF-1 transgene exerts its effects in an autocrine or paracrine manner, circumventing the adverse side effects of systemic IGF-1 administration. Expression of the mIGF-1 transgene safely enhanced and preserved muscle fiber integrity even at advanced ages (Musarò *et al.,* 2001), suggesting that the MLC/mIGF-1 transgene acts as a survival factor by prolonging the regenerative potential of younger muscle.

The capacity of the mIGF-1 transgene to attenuate the structural and functional consequences of muscle aging was independent of its action during embryogenesis or early postnatal life, since local delivery of mIGF-1 in individual mouse muscles by AAV virus mediated gene transfer also permanently blocked age-related loss of muscle size and strength, presumably by improving regenerative capacity (Barton-Davis *et al.,* 1998) through increases in satellite cell activity. Because it is clear that IGF-1 can prevent aging- related loss of muscle function (Barton-Davis *et al.,* 1998; Musarò *et al.,* 2001), it is possible that IGF-1 can prevent or diminish muscle loss associated with disease.

To prove this hypothesis, we introduce mIGF-1 into the mdx dystrophic animals (mdx/mIGF-1).

This approach allowed for the assessment of the maximum potential benefit that could be derived from IGF-1 expression for dystrophic muscle, as well as examination of both the diaphragm and the extensor digitorum longus (EDL), which display a spectrum of dystrophic pathologies. By analyzing

both muscle morphology and function in transgenic mdx/mIGF-1 we observed a significant improvement in muscle mass and strength, a decrease in myonecrosis, and a reduction in fibrosis in aged diaphragms (Barton *et al.,* 2002). In particular, even though IGF-1 has been shown to stimulate fibroblasts, there is a net decrease in fibrosis in the diaphragm of the mdx/mIGF-1 mice. In fact, age-related fibrosis in the mdx diaphragm was effectively eliminated by mIGF-1 expression. It may be that the efficient and rapid repair of the mdx/mIGF-1 muscles prevents the establishment of an environment into which the fibroblasts migrate. This is of particular relevance to the human dystrophic condition where virtually all skeletal muscles succumb to fibrosis (Louboutin *et al.,* 1993; Morrison *et al.,* 2000). Thus, the results found in the mouse diaphragm suggest that IGF-1 may be effective not only in increasing muscle mass and strength, but also in reducing fibrosis associated with the disease.

Finally, signaling pathways associated with muscle regeneration and protection against apoptosis were significantly elevated. These results suggest that a combination of promoting muscle regenerative capacity and preventing muscle necrosis could be an effective treatment for the secondary symptoms caused by the primary loss of dystrophin.

More recently, we reported a protective effects of muscle-restricted mIGF-1 against the dominant action of mutant SOD1G93A gene involved in the progression of a neurodegenerative disease, known as Amyotrophic Lateral Sclerosis (Dobrowolny *et al.,* 2005). Muscle-restricted expression of a localized IGF-1 isoform maintained muscle integrity and enhanced satellite cell activity in SOD1G93A transgenic mice, inducing calcineurin-mediated regenerative pathways. Muscle-specific mIGF-1 expression also stabilized neuromuscular junctions, reduced inflammation in the spinal cord, and enhanced motor neuronal survival, delaying the onset and progression of the neuromuscular disease.

These data suggest that IGF-1 is critical in mediating muscle growth and its loss appears central to muscle atrophy in muscle pathologies.

The anabolic effects of IGF-1 may be due in part to stimulation of activation of satellite cells that have a precocious ability to form myotubes compared to those isolated from wild–type littermates, and in part to the modulation of the tissue niche, creating a qualitative environment to efficiently sustain muscle regeneration and repair (Pelosi *et al.,* 2007). It is not known whether in transgenic animals, the satellite cells have an increased ability for self-renewal or whether there is an increased recruitment of non-satellite cells. Our recent experimental evidences indicate that IGF-1 promotes the two suggested pathways which can be considered two temporally separated events of the same biological process. We demonstrated that upon muscle injury, stem cells expressing c-Kit, Sca-1, and CD45 antigens increased locally and the percentage of the recruited cells were conspicuously enhanced by IGF-1 expression (Musarò *et al.,* 2004).

These results establish mIGF-1 as a potent enhancer of stem cell-mediated regeneration and provide a baseline to develop strategies to improve muscle regeneration in muscle diseases.

The novel role of neurohypophyseal hormones in muscle development and homeostasis

Since this topic has emerged in recent years due to the work of our and other laboratories, and has never been the subject of a review, relevant findings will be summarized here in some detail.

Until the early 1990s neurohypophyseal hormones (vasopressin, AVP, acting on blood vessels, kidney and CNS; oxytocin, OT, acting on uterus and mammary gland) were not particularly known for effects on skeletal muscle. Wakelam *et al.* had shown an indeed modest effect of AVP on carbohydrate metabolism in muscle fibers (Wakelam *et al.,* 1982), and the presence of functional AVP receptors in the rat myogenic L6 cell line had been reported (Wakelam *et al.,* 1987).

Biological effects of vasopressin and oxytocin on skeletal muscle

Addition of AVP (and, with a lower sensitivity, of OT) to the culture medium of L6 and L5 myoblasts and of satellite cells resulted in a significant increase of the percentage of fusion and in the formation of hypertrophic myotubes compared to controls, in the absence of significant effects on cell proliferation. Both early (Myf-5 and myogenin) and late (myosin, acetylcholine receptor subunits) myogenic differentiation markers were stimulated by AVP in a structure- and concentration- dependent fashion (Nervi *et al.,* 1995). By setting up an efficient serum-free culture medium for L6 and L5 myoblasts and for mouse satellite cells we could demonstrate that AVP effectively induced myogenic

differentiation in the absence of other factors, allowing us to conclude that terminal myogenic differentiation requires the presence of differentiation factors rather than the absence of growth factors. In addition AVP and any of the IGFs induced maximal stimulation of differentiation when co-administered to the cultures, indicating that the two factors activated (at least partially) distinct signaling pathways (Minotti *et al.,* 1998). These findings led us to propose that AVP (or a still unidentified analog) may represent a novel physiological modulator of skeletal muscle differentiation. This hypothesis was also supported by data indicating that both in human and in mouse embryonic and fetal muscles high levels of immuno-reactive AVP can be detected (Smith *et al.,* 1992;Naro *et al.,* 1994), and by the report of the presence of a vasopressin-like peptide in the mammalian sympathetic nervous system (Hanley *et al.,* 1984).

Signaling of neurohypophyseal hormones in muscle cells

We investigated the intracellular signals elicited by AVP in several clones of L6 and L5 cells, in rat satellite cells and in chick embryo myoblasts, showing that AVP induces concentration-dependent (0.1 nM - 1 μM) stimulation of phospholipase C (PLC) activity and regulates the intracellular pH with mechanisms involving Na^+ and anion transport across the plasma membrane. Inositol 1,4,5-trisphosphate production was maximally stimulated within 2 - 5 sec of treatment with AVP, immediately followed by release of Ca^{2+} from intracellular stores. Activation of protein kinase C as well as administration of antagonists competing with AVP for binding at V1 receptors inhibited the responses. Interestingly, the responsiveness of different L6 clones to AVP positively correlated with their myogenic potential (Teti *et al.,* 1993).

AVP stimulation of myogenic cells also results in the activation of phospholipase D (PLD) - dependent phosphatidylcholine (PtdCho) breakdown. AVP induces the monophasic generation of phosphatidic acid (PA) and the biphasic increase of sn-1,2-diacylglycerol (DAG), consisting in a rapid peak (within 5 sec of AVP treatment, resulting from PLC activity), followed by a sustained phase (peaking at 2 min, dependent upon PtdCho-PLD activity and PA dephosphorylation) (Naro *et al.,* 1997). PLD activation is elicited at AVP concentrations (EC_{50} = 0.4 nM) two orders of magnitude lower than those required for PLC activation (EC_{50} = 50 nM). Interestingly, the dose-dependency of myoblast fusion (EC_{50} = 0.3 nM) is superimposable to that of PLD activity, indicating an important role of PLD in the mechanism of AVP-induced muscle differentiation. Actually, the AVP-dependent stimulation of PtdCho breakdown in myoblasts is so intense that it significantly alters the plasma membrane environment and the membrane exchange dynamics. PC-PLD activation in AVP-stimulated L6 myogenic cells is accompanied by decreased membrane fluidity and increased exocytosis which, coupled to PC *de novo* synthesis, restores plasma membrane-PtdCho, conspicuously consumed during PLD-mediated signal transduction (Coletti *et al.,* 2000b; Coletti *et al.,* 2000a).

In addition to an obvious cross-talk between PLD and PLC signaling pathways, AVP signals interfere with the cAMP system in myoblasts. It is well known that cAMP-dependent protein kinase (PKA) negatively regulates myogenic differentiation by inhibiting the activity of myogenic Helix-Loop-Helix transcription factors (Li *et al.,* 1992;Winter *et al.,* 1993). In addition PA, conspicuously produced upon PLD activation, selectively stimulates the activity of specific cAMP-phosphodiesterase isoforms (Némoz *et al.,* 1997). In L6 myoblasts we observed that AVP stimulation caused a rapid increase of PDE4 activity which remained elevated for 48 h. In the continuous presence of vasopressin, cAMP levels and PKA activity were lowered, thus allowing the nuclear translocation and the transcriptional activity of myogenesis regulatory factors (Naro *et al.,* 1999; Naro *et al.,* 2003). It is worth noting that the IGFs do not possess by themselves (in the absence of serum or other factors) the ability to significantly modulate PDE activity and this represent a major difference in the signaling pathway elicited by the two classes of factors (De Arcangelis *et al.,* 2003)

At the nuclear level, the AVP signaling in myogenic cells, relying on the activation of both Ca^{2+} /calmodulin dependent kinase and calcineurin, induces the nuclear export of histone deacetylase 4 (known to negatively interact with the activity of myocyte enhancer factor-2 (MEF2) (Miska *et al.,* 2001), increased expression and transcriptional activity of MEF2 and, downstream of this, increased expression of myogenin and Myf-5 (Scicchitano *et al.,* 2002). In addition, the formation of multifactor complexes, required for the full

expression of the differentiated phenotype, occurs in AVP-stimulated myoblasts: MEF2–NFATc1 complexes appear to regulate the expression of early muscle-specific gene products such as myogenin, while the activation of muscle-specific gene expression characteristic of late differentiation involves the formation of complexes including also GATA2 (Scicchitano *et al.,* 2005).

Receptors for neurohypophyseal hormones in muscle

Neurohypophyseal hormones target cells express at least one of a family of receptors which include three AVP receptor subtypes and one OT receptor, all members of the seven transmembrane domain, G-protein coupled receptor superfamily and sharing a high degree of homology both at the gene and at the protein level (Barberis *et al.,* 1998). V1a and V1b AVP receptors, and the OTR, are functionally coupled to PLC and PLD via Gq/11, whereas the V2 AVP receptor is functionally coupled to adenylate cyclase.

Both undifferentiated and differentiated L6 myogenic cells express V1aR as the only member of this receptor family (Naro *et al.,* 2003; Alvisi *et al., submitted*). V1aR is also expressed in human skeletal muscle, whereas OTR expression seems to prevail in the rat (Thibonnier *et al.,* 1996; Alvisi *et al., submitted*). Human satellite cells have been recently reported to express the OTR (Breton *et al.,* 2002), and mouse satellite cells appear to express both the OTR and the V1aR (Alvisi M., *personal communication*).

Physiological role of neurohypophyseal hormones on muscle development and homeostasis

Indeed the above data indicate that AVP and OT are potent inducers of myogenic differentiation and hypertrophy in myogenic cell lines and satellite cells. The expression of receptors for these hormones in developing and adult muscle and in satellite adds to the physiological relevance of these data. It is particularly interesting that several reports indicate that muscular exercise results in a significant increase of circulating AVP, both in human and in other mammals, thus posing the theoretical basis for the physiological regulation of muscle hypertrophy by neurohypophyseal hormones (Melin *et al.,* 1980; Convertino *et al.,* 1981; Alexander *et al.,* 1991; Melin *et al.,* 1997). Furthermore the calcineurin pathway, which is strongly stimulated by AVP, was shown to be essential for muscle regeneration in normal and dystrophic animals (Stupka *et al.,* 2004); and determination of muscle specificity, an important factor in muscle development, is finely regulated by SM22, which in turn is regulated by AVP (Chang *et al.,* 2001; Kaplan-Albuquerque *et al.,* 2003).

In conclusion, the hypothesis that neurohypophyseal hormones play important physiological roles in skeletal muscle development and homeostasis is gaining support by a wide body of evidence and requires further investigation.

Inhibitory signals affecting the myogenic potential of muscle precursor cells

The relevance of the niche in conditioning the myogenic potential of muscle precursor cells during muscle regeneration is apparent from the above. Muscle regeneration is affected by a wide range of environmental signals highly variable not only timewise but also depending on the physiological or pathological conditions of the musculature. Several cytokines and other factors, such as IL-1, IL-6, TNF-α, and IFN-γ have been proven to negatively affect muscle differentiation both *in vitro* and *in vivo* (Miller *et al.,* 1988; Coletti *et al.,* 2002; Guttridge *et al.,* 2000). Elevated levels of cytokines, associated to chronic inflammation, are observed in several chronic diseases, ranging from cancer to AIDS, and from chronic heart failure to kidney disease. In these condition a severe form of muscle wasting often occurs, named cachexia (Tisdale, 2002; Argiles *et al.,* 1999). Guttridge and coworkers have recently shown that cancer cachexia is associated to skeletal muscle damage resulting from deregulation of the dystrophin glycoprotein complex (Acharyya *et al.,* 2005). We have reported that cachexia is associated to diminished muscle regeneration following experimentally induced injury (Coletti *et al.,* 2005). Collectively, this evidence suggests a model whereby the damaged skeletal muscle activates reparative pathways involving satellite and myogenic stem cells. Based on all the above muscle atrophy may actually result from a combined process of muscle protein reduction, muscle fiber death and attenuated muscle regeneration.

In this context it has become urgent to understand the molecular mechanisms underlying the response of muscle precursor cells to cytokines inducing cachexia and to other inhibitory signals which could hamper muscle regeneration.

Among the inducers of cachexia TNF-α is probably the most studied in relation to its regulatory effects on muscle differentiation. It is established that TNF-α downregulates the myogenic factors MyoD and myogenin (Szalay *et al.,* 1997) through a not fully characterized mechanism involving NF-kB (Guttridge *et al.,* 2000). Our contribution to this topic revealed a novel role for caspases in mediating the block of muscle differentiation observed in the presence of TNF-α. We have shown that a Bax- and PW1/Peg3-dependent activation of caspase pathways occurs upon TNF-α stimulation in myogenic cells, and that caspase activity is necessary for the block of differentiation to occur (Coletti *et al.,* 2002). PW1 had been implicated previously in p53-mediated apoptosis and Bax activation in non-muscle cells (Relaix *et al.,* 2000). We showed that PW1 is necessary to recruit p53-dependent caspase pathways to a negative regulation of muscle differentiation in the presence of TNF-α (Coletti *et al.,* 2002). PW1 expression in developing, adult and regenerating muscle, as well as in stem cells and myogenic cell lines, makes this protein a very intriguing candidate for the regulation of muscle precursor cell fate.

Using a novel *in vivo* model of cachexia we extended our previous observation, confirming that TNF-α inhibits myogenesis during the adult life (Coletti *et al.,* 2005). In this work we induced muscle wasting specifically due to TNF-α by overexpressing a secreted, circulating form of murine TNF-α by electroporation-mediated gene delivery to skeletal muscle. In this context we reported that the hallmarks of muscle regeneration following freeze injury were significantly reduced, indicating a bona fide compromised muscle homeostasis (Coletti *et al.,* 2005).

The relevance of the findings above stems from the fact that PW1 regulates muscle response to cytokines both *in vitro* and *in vivo* in concert with p53 (Schwarzkopf *et al.,* 2007). We reported that $p53^{-/-}$ mice are less sensitive to cancer cachexia and that overexpressing a truncated dominant negative form of PW1 (Δ-PW1) in skeletal muscle fibers protects them from atrophy induced by tumor load. Interestingly, both PW1 and p53 are necessary for the TNF-α inhibitory effects on muscle differentiation *in vitro* to occur. In fact, ablation of p53 expression either genetically or chemically makes the myogenic cells resistant to TNF-α-mediated inhibition of differentiation. p53 is expressed in muscle stem cells and colocalizes with PW1 in regenerating muscle fibers. Accordingly, PW1 and p53 seem to participate in a positive regulatory feedback whereby they regulate each other expression (Schwarzkopf *et al.,* 2007).

All together these observations support the hypothesis that muscle stem cells are critical for muscle homeostasis both in physiological and pathological conditions (such as cachexia), although the mechanisms of how perturbation of stem cells triggers muscle atrophy remains unresolved.

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Anatomy of emotion: a 3D study of facial mimicry

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Alterations in facial motion severely impair the quality of life and social interaction of patients, and an objective grading of facial function is necessary. A method for the non-invasive detection of 3D facial movements was developed. Sequences of six standardized facial movements (maximum smile; free smile; *surprise* with closed mouth; *surprise* with open mouth; right side eye closure; left side eye closure) were recorded in 20 healthy young adults (10 men, 10 women) using an optoelectronic motion analyzer. For each subject, 21 cutaneous landmarks were identified by 2-mm reflective markers, and their 3D movements during each facial animation were computed. Three repetitions of each expression were recorded (within-session error), and four separate sessions were used (between-session error). To assess the within-session error, the technical error of the measurement (random error, TEM) was computed separately for each sex, movement and landmark. To assess the between-session repeatability, the standard deviation among the mean displacements of each landmark (four independent sessions) was computed for each movement. TEM for the single landmarks ranged between 0.3 and 9.42 mm (intra-session error). The sex- and movement-related differences were statistically significant (two-way analysis of variance, p=0.003 for sex comparison, p=0.009 for the six movements, p<0.001 for the sex x movement interaction). Among four different (independent) sessions, the left eye closure had the worst repeatability, the right eye closure had the best one; the differences among various movements were statistically significant (one-way analysis of variance, p=0.041). In conclusion, the current protocol demonstrated a sufficient repeatability for a future clinical application. Great care should be taken to assure a consistent marker positioning in all the subjects.

Key words: 3D, motion analysis, mimics.

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Bones, muscles, cutaneous and subcutaneous layers all contribute to a unique facial morphology in the single individual (Vidarsdottir *et al.,* 2002). This morphology is never static, but it continuously acts and reacts to environmental and internal stimuli. The face plays a major role in social communication and interaction (Hennessy *et al.,* 2005; Johnson and Sandy, 2003; Matoula and Pancherz, 2006; Nooreyazdan *et al.,* 2004; Tarantili *et al.,* 2005), and it carries information that allows the identification of a single person (DeCarlo *et al.,* 1998; Fraser *et al.,* 2003; Shi *et al.,* 2006).

Functional impairments in facial expression may be caused by central nervous system diseases (Parkinson disease), facial nerve paralysis, dentofacial deformities and scars, congenital anomalies like cleft lip (Linstrom, 2002; Linstrom *et al.,* 2002; Mishima *et al.,* 2004; Nooreyazdan *et al.,* 2004; Tarantili *et al.,* 2005; Trotman *et al.,* 1998a; Wachtmann *et al.,* 2001), and may provoke serious alterations in the quality of life of the patients. Additionally, modifications in facial motion had been reported in patients affected by several psychiatric disorders (Mergl *et al.,* 2005).

Several qualitative, subjective methods for grading facial function have been developed for clinical applications, as recently reviewed (Linstrom, 2002). Their principal limitation is the reduced inter-observer agreement (Linstrom, 2002). In contrast, a quantitative method for the assessment of facial movements could help in diagnosis, treatment planning, and post-treatment follow-up (Linstrom, 2002; Linstrom *et al.,* 2002; Trotman *et al.,* 2000; Tzou *et al.,* 2005). In the past, static (photographic) and dynamic (cinematographic) two-dimensional systems had been devised and applied in several clinical contests (Linstrom, 2002; Linstrom *et al.,* 2002; Tarantili *et al.,* 2005; Wachtmann *et al.,* 2001). Unfortunately, facial motion is a complex activity that develops in all three spatial planes;

two-dimensional recordings can significantly underestimate facial movements (Frey *et al.,* 1999; Gross *et al.,* 1996; Nooreyazdan *et al.,* 2004).

Currently, various three-dimensional motion analyzers allow a non-invasive quantitative assessment of soft tissue facial movements without interfering with the subject, and details about instruments, measurement protocols and data analysis have been reported (Frey *et al.,* 1999; Giovanoli *et al.,* 2003; Johnston *et al.,* 2003; Mergl *et al.,* 2005; Mishima *et al.,* 2004; Nooreyazdan *et al.,* 2004; Weeden *et al.,* 2001).

Previous investigations developed standardized sequences of facial animations, but only symmetric movements had been studied so far. In contrast, asymmetric motions could allow a better assessment of unilateral facial palsy, for instance after facial nerve lesion. Additionally, repeatability of movements had seldom been reported (Johnston *et al.,* 2003; Trotman and Faraway, 1998; Trotman *et al.,* 1998a; Weeden *et al.,* 2001). Indeed, in all measuring systems the minimal *noise* level should be estimated, and used as a base to detect actual variations between and within individuals. For ethical and practical reasons, repeatability should be assessed with healthy, non-patient subjects; this could also obtain a normal, reference data base for the subsequent comparison of patients.

The aim of the present study was to develop a method for the non-invasive detection of three-dimensional facial movements. An optoelectronic motion analyzer was used to record sequences of standardized facial symmetric and asymmetric movements. A measurement protocol was devised, intra-session and inter-session repeatability were assessed in healthy volunteers, and reported in the current investigation.

Materials and Methods

Subjects

Twenty healthy young adults (10 men and 10 women) aged 20 to 30 years were measured. The subjects were recruited from the students and staff attending the Department of Human Morphology of Milan University. All subjects had a clinically normal facial function, no previous facial trauma, paralysis or surgery, no known neurological diseases. They had no current orthodontic treatment and no facial hair that would interfere with marker placement.

After the nature and possible risks of the study had been completely described, written informed consent was obtained from each participant. The protocol used in the current study was approved by the local Ethics Committee, and it did not involve dangerous or painful activities.

Data collection

Facial movements were recorded using an optoelectronic three-dimensional motion analyzer operating at 60 Hz (SMART System, E-motion, Padova, Italy). The method has been described in detail elsewhere (Ferrario *et al.,* 2002, 2005; Sforza *et al.,* 2002, 2003). In brief, six high-resolution infrared sensitive charge-coupled device video cameras coupled with a video processor defined a working volume of 44 (width) cm × 44 (height) cm × 44 (depth) cm; metric calibration and correction of optical and electronic distortions were performed before each acquisition session, with a resulting mean dynamic accuracy of 0.121 mm (SD 0.086), corresponding to 0.0158% (Capozzo *et al.,* 2005).

The subject was positioned inside the working volume sitting on a stool without backrest, and was asked to perform a series of standardized facial expressions. During the execution of the movement, for any TV camera special software recognized the coordinates of the center of gravity of 21 passive markers positioned on facial landmarks (Figure 1). Subsequently, all the coordinates were converted to real metric data, and a set of *x-, y-, z*-coordinates for each landmark in each frame that constituted each expression was obtained.

For each subject, 2-mm reflective markers were located on the following 21 anatomical landmarks (Ferrario *et al.,* 2003; Nooreyazdan *et al.,* 2004): tr, trichion; n, nasion; prn, pronasale; ls, labiale superius; sl, sublabiale; pg, pogonion; sci, right and left superciliare; ex, right and left exocanthion; or, right and left orbitale; ac, right and left nasal alar crest; ch, right and left cheilion; li, right and left lower lip points halfway between cheilion and sublabiale; t, right and left tragion; v, vertex (Figure 1). Markers' positions were carefully controlled to avoid any interference with facial movements; bi-adhesive plaster was used to position the markers on the skin.

Markers t_r, t_l and v defined a cranial plane of reference that was used to eliminate head movements during facial animations; the plane of reference was

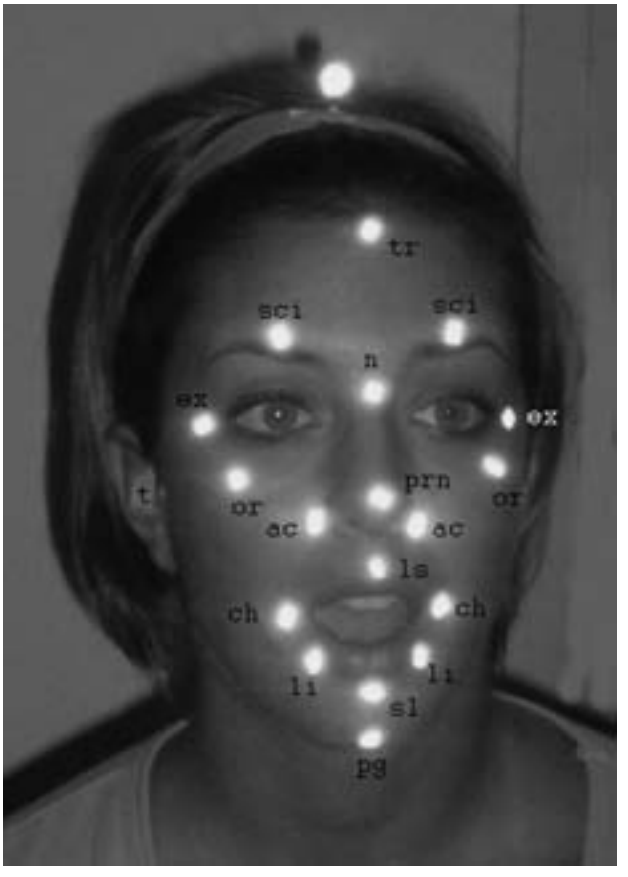

Figure 1. Soft tissue markers used for the analysis of facial movements.

also used to standardize head position within and between subjects.

Each subject was instructed to perform six standardized, maximum facial animations from rest:

- instructed (maximum) smile (bite on the back teeth, smile as much as possible, and then relax);
- free (natural) smile;
- *surprise* with closed mouth (bite on the back teeth, make a surprise expression without opening the mouth, with a prevalent movement of the forehead and eyes);
- *surprise* with open mouth (make a surprise expression opening the mouth, with a global facial movement);
- right side eye closure (maximal closure of the eye);
- left side eye closure (maximal closure of the eye);

Each subject was allowed to practice before actual data acquisition; three repetitions of each expression were then recorded for each subject without modifications of the marker positions. The entire recording session lasted approximately 20 minutes. One man was assessed in four separate occasions (sessions); in each session, the markers were repositioned on his face. In each session, three repetitions of the movements were performed, and mean values computed.

Data analysis

As detailed by Ferrario *et al.* (2005), the patient's head and neck movements were subtracted from the raw facial movements using the three cranial markers, and only movements occurring in the face (activity of mimic muscles, mouth opening during the *surprise with open mouth* expression) were further considered. Subsequently, for each of the 18 facial markers, the three-dimensional movements during each facial animation were computed. The origin of axes was set in the nasion.

In this first report, only the modulus (intensity) of the three-dimensional vector of maximum displacement from rest will be further considered, neglecting the trajectory followed by each marker during motion.

To assess the differential movements between the two hemi-faces, percentage indices of asymmetry were computed as: (right displacement – left displacement) / (right displacement + left displacement) x 100; in particular, markers sci_r, ex_r, or_r, and markers sci_l, ex_l, or_l gave the eye asymmetry index; markers ac_r and ac_l gave the nose asymmetry index; markers ch_r, li_r, and ch_l, ls_l gave the mouth asymmetry index. The indices range between –100 (complete left side prevalence during the movement) and 100 (complete right side prevalence).

Finally, the total facial movement was obtained as the vectorial sum of the movement of the 18 facial markers: the larger the value, the larger the facial movement.

Statistical calculations

To assess the within-session repeatability of each movement, the technical error of the measurement (random error, $TEM = [\Sigma (D^2)/ 2 \times N]^{0.5}$, where D is the difference between the two repeated measurements, and N is the number of subjects) was computed separately for each sex, movement and landmark. The calculations were performed also for the asymmetry indices and the total mobility. To compare the landmark TEMs, a two-way factorial analysis of variance with replicates was run (factor 1, sex; factor 2, movement; the sex x movement interaction was also computed). *Post-hoc* tests

Table 1. Intra-session repeatability. Technical error of measurement for single landmarks (mm), total mobility (mm), and asymmetry indices (%).

	Max smile		*Free smile*		*Surprise- closed m.*		*Surprise- open m.*		*R. eye closure*		*L. eye closure*	
	F	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*
Sci R	1.65	1.53	3.30	2.13	3.40	0.65	2.35	7.40	2.95	1.06	0.61	0.88
Ex R	1.76	1.96	3.08	1.53	1.83	1.19	9.47	1.31	1.73	0.65	0.89	1.90
Or R	0.73	2.19	3.51	1.70	1.14	0.66	1.48	1.51	0.86	0.78	0.50	0.79
Sci L	1.10	0.83	3.97	2.04	3.37	0.93	1.85	3.22	3.08	1.53	0.62	0.55
Ex L	1.56	0.80	4.31	2.03	3.64	0.54	1.44	7.69	7.75	1.36	0.46	0.67
Or L	0.73	2.24	4.52	1.59	3.46	0.30	3.71	0.78	2.65	2.39	0.82	0.96
Tr	0.83	1.00	3.67	1.72	3.34	0.50	2.91	0.86	2.10	1.02	0.56	0.57
N	0.84	1.48	3.28	1.85	3.72	0.64	5.61	2.04	2.31	1.22	0.37	0.64
Prn	1.49	1.50	3.84	1.56	3.33	0.74	1.24	1.12	2.13	0.76	0.75	3.63
Ac R	1.50	2.26	4.01	1.92	4.50	1.17	1.90	1.26	1.93	1.33	0.71	4.42
Ac L	2.48	2.25	3.55	0.92	3.65	0.55	3.64	0.82	3.49	0.85	4.58	1.49
Ls	0.93	5.73	3.44	0.53	2.97	0.51	2.48	1.11	1.37	2.40	2.60	1.56
Sl	1.04	2.41	2.06	2.26	2.92	0.44	4.39	3.19	4.43	2.45	4.19	1.28
Pg	0.73	2.33	2.51	2.47	3.45	0.74	3.19	3.35	3.16	3.08	1.28	1.13
Ch R	0.92	3.44	1.88	1.57	3.39	0.83	1.72	2.76	1.25	2.59	1.31	1.46
Ch L	1.19	3.88	1.75	1.50	3.27	0.47	1.85	2.03	3.34	1.55	1.43	3.13
Li R	0.69	2.39	3.19	1.73	3.02	0.46	3.80	6.61	3.25	2.00	4.87	1.47
Li L	1.18	3.32	2.59	1.91	3.06	1.64	2.61	6.12	3.49	3.46	4.25	2.06
Mean	1.19	2.31	3.25	1.72	3.19	0.72	3.09	2.95	2.85	1.69	1.71	1.59
SD	0.48	1.21	0.81	0.46	0.72	0.33	1.97	2.37	1.54	0.85	1.61	1.10
Total mobility	12.61	19.44	60.78	20.34	23.55	9.04	16.64	18.21	36.11	19.18	21.86	15.41
Asymmetry eye	9.41	10.39	12.60	11.18	10.88	2.91	9.62	16.56	20.20	9.47	15.64	6.28
Asymmetry nose	9.51	12.40	16.63	10.95	14.70	4.23	14.22	9.50	17.06	6.20	17.57	9.19
Asymmetry mouth	7.70	10.33	7.43	4.05	8.49	2.21	2.96	6.36	18.05	13.74	13.71	4.65

were performed by unpaired Student's t tests, with a Bonferroni correction for multiple testing.

For each movement, the between-session repeatability was assessed by calculating the standard deviation among the mean displacements of each landmark (four independent sessions). The same calculations were performed for the asymmetry indices and the total mobility. Landmark repeatability in the various movements was compared by one-way analysis of variance, followed by *post-hoc* tests (Tukey's honestly significant difference).

The level of significance was set at 5% for all statistical analyses.

Results

Intra-session repeatability for the six analyzed movements is reported in Table 1. The technical errors of measurement for the single landmarks ranged between 0.3 (left orbitale landmark in the *surprise* movement in men) and 9.42 mm (right exocanthion in the *surprise with open mouth* movement in women). Overall, men were more repeatable than women in all movements except the *maximum smile*. The sex- and movement- related differences were statistically significant (two-way analysis of variance, p=0.003 for male-female comparison, p=0.009 for the six movements, p<0.001 for the sex x movement interaction); in particular, the *maximum smile*, *free smile* and *surprise* movements were significantly different between sexes (post-hoc unpaired Student's t tests).

The total mobility indices had a repeatability similar to that found for the single landmarks, with TEMs ranging between 9.04 and 60.78 mm (average TEM per landmark 0.5-3.38 mm). Repeatability of the asymmetry indices was sex- and expression-specific: for instance, in the right and left eye closure, women were more repeatable in their asymmetry than men. In the *surprise* animation, asymmetry was more repeatable in men than in women; similar male and female values were found for the *maximum smile*. Overall, in both sexes mouth asymmetry was more reproducible than nose and eye asymmetry.

Among four different (independent) sessions, the left eye closure had the worst repeatability (Table 2). The best repeatability was found for the right eye closure (single landmark standard deviations up to 1.05 mm, total mobility index 2.56 mm). In the *maximum smile* and *surprise with open mouth* movements, the lip landmarks had the largest standard deviations (least repeatability). The differences among various movements were statistically significant (one-way analysis of variance on the standard deviations of the single landmarks, p= 0.041); *post-hoc* tests found that the left eye closure was significantly different from the other movements.

Overall, the asymmetry indices computed for the *surprise* movement were the most repeatable, while

Table 2. Inter-session repeatability. Standard deviation of the mean displacements (four independent sessions) for single landmarks (mm), total mobility (mm), and asymmetry indices (%).

	Max smile	*Free smile*	*Surprise- closed m.*	*Surprise- open m.*	*R. eye closure*	*L. eye closure*
Sci R	0.86	1.52	1.18	0.65	0.22	1.87
Ex R	0.56	1.80	0.13	0.23	0.87	1.03
Or R	0.85	1.32	0.30	0.50	0.68	1.36
Sci L	0.56	0.47	0.22	0.32	0.30	1.68
Ex L	0.44	0.16	0.22	0.27	0.30	0.40
Or L	0.50	0.47	0.40	0.59	0.26	0.85
Tr	0.63	0.24	0.15	0.43	0.09	1.84
N	0.85	0.37	0.36	0.36	0.18	2.13
Prn	1.05	0.41	0.59	1.11	0.10	2.36
Ac R	2.51	0.52	0.49	0.57	0.50	1.25
Ac L	0.82	0.38	1.30	0.50	1.05	1.29
Ls	0.47	0.23	0.61	0.66	0.33	1.83
Sl	0.86	0.85	0.61	1.76	0.67	2.30
Pg	0.31	0.96	0.78	1.53	0.25	2.36
Ch R	0.85	1.39	0.52	0.60	0.87	1.33
Ch L	1.12	0.69	0.67	0.69	0.51	2.62
Li R	3.54	1.12	0.56	1.10	0.53	1.37
Li L	1.70	0.66	0.69	1.28	0.20	2.39
Mean	1.03	0.75	0.54	0.73	0.44	1.68
SD	0.81	0.49	0.32	0.44	0.29	0.61
Total mobility	10.23	5.47	9.43	10.68	2.56	31.80
Asymmetry eye	7.07	9.92	1.01	1.97	1.53	12.90
Asymmetry nose	10.11	5.02	7.13	15.12	9.58	9.65
Asymmetry mouth	14.13	4.89	1.24	0.55	2.27	2.81

those computed for the *maximum smile* were approximately three times more variable. In the four different sessions, nose asymmetry was very variable, especially for the two *surprise* movements. Eye asymmetry was more repeatable for the right eye closure (SD 1.53%) than for the contralateral left eye closure (SD 12.9%). Repeatability of lip asymmetry was very low for the *maximum smile* movements (SD 14.13%).

Figure 2 shows the start (rest position) and end (maximum displacement) frames of one *surprise with open mouth* movement performed by one of the analyzed subjects. The vectors of maximum displacement from rest are also shown for each of the 18 facial landmarks.

Discussion

The non-invasive detection, recording and quantitative analysis of three-dimensional facial movements is an important step for the objective description of facial morphology and function. Alterations in facial motion severely impair the quality of life and social interaction of patients (Nooreyazdan *et al.,* 2004; Tarantili *et al.,* 2005), and the objective grading of facial function is a key step for the diagnosis, treatment and follow-up of several disorders (Linstrom, 2002).

Among the various instruments developed for the assessment of facial movements, optoelectronic motion analyzers working with passive, retroreflective markers appear the best suitable for the collection of data in both patients and healthy, non-patient individuals. They allow a complete and detailed assessment of motion in all parts of the face; qualitative and quantitative data can be compared between and within individuals (Coulson *et al.,* 2002; Johnston et al., 2003; Mishima *et al.,* 2004; Nooreyazdan *et al.,* 2004; Trotman and Faraway, 1998, 2004; Trotman *et al.,* 1998b; Weeden *et al.,* 2001).

In the current study, an optoelectronic motion analyzer was used to record a standardized set of facial symmetric and asymmetric movements. Facial movements were detected by using passive markers glued on the face. These markers are small and practically weightless, allowing the detailed analysis of all facial features without interfering with the movement (Lundberg, 1996). The method has already been successfully used by other investigators (Coulson *et al.,* 2002; Mishima *et al.,* 2004; Nooreyazdan *et al.,* 2004; Trotman and Faraway, 1998; Trotman *et al.,* 1998a, b; Weeden *et al.,* 2001). Alternative protocols marked the landmarks directly on the face using an eyeliner pencil (Frey *et al.,* 1999; Giovanoli *et al.,* 2003; Johnston *et al.,* 2003; Tzou *et al.,* 2005). In both cases, the markers have to be tracked semi-automatically for their three-dimensional reconstruction. In other applications, the facial features of interest were automatically singled out without previous marking

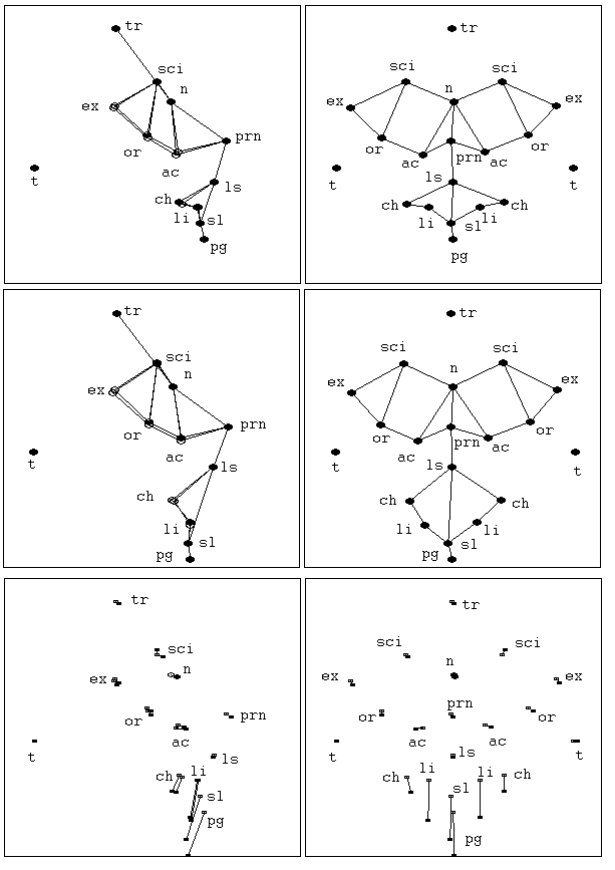

Figure 2. Start (rest position, upper panel) and end (maximum displacement, middle panel) frames of one *surprise with open mouth* movement. The vectors of maximum displacement from rest are also shown for each of the 18 facial landmarks (lower panel). Frontal (right side) and lateral (left side) views of the face.

(Mishima *et al.,* 2004; Wachtmann *et al.,* 2001). Automatic detection is likely to be faster than the use of physical markers, but it necessitates a careful control of experimental conditions; additionally, it may be of difficult application in patients with facial scars or with hairs and nevi. Also, a dark complexion may obtrude the digitization (Majid *et al.,* 2005).

The number of markers used in the current investigation is well comparable to those reported in previous studies, that ranged between 15-20 (Coulson *et al.,* 2002; Frey *et al.,* 1999; Giovanoli *et al.,* 2003; Johnston *et al.,* 2003; Tzou *et al.,* 2005) and 30-34 (Nooreyazdan *et al.,* 2004; Trotman *et al.,* 1998b; Weeden *et al.,* 2001). Marker number should be a compromise between accurate detection of facial movements, time for positioning and processing, and actual anatomical and functional significance. Indeed, while an increased number of markers may allow a more detailed assessment of motion (Nooreyazdan *et al.,* 2004), their application on the patient's face could be cumbersome, and their semiautomatic tracking long, tedious and more prone to error. Also, the correspondence between the markers and the anatomical landmarks could be lost if markers are positioned not only on facial landmarks but also between landmarks (Nooreyazdan *et al.,* 2004). Any lack of correspondence makes intra-subject (longitudinal) and inter-subject (cross-sectional) analyses of difficult biological significance because only the use of landmarks with a clear definition (by either inspection or palpation) allows to reposition the markers in the same anatomical loci.

Marker dimensions should also be chosen to allow a unique identification by the motion analysis system within the working volume (Cappozzo *et al.,* 2005; Sforza *et al.,* 2003): too small markers may not be clearly detected from the background noise, but large markers do not allow a detailed analysis of the characteristics of facial movements. Overall, dimensions between 2 (Nooreyazdan *et al.,* 2004; Trotman and Faraway, 2004) and 7 mm (Coulson *et al.,* 2002) have been used so far.

A general limitation of three-dimensional non-invasive motion analyses is skin movement (Leardini *et al.,* 2005). Usually, external, soft-tissue markers are used to approximate internal (bones and joints) motions, which position is estimated with more or less complex algorithms (Ferrario *et al.,* 2002, 2005; Leardini *et al.,* 2005; Sforza *et al.,* 2002, 2003). In all these applications, markers must be positioned in body areas where the subcutaneous tissues do not allow large movements between the skeleton and the skin (Leardini *et al.,* 2005). In contrast, in the current study no hard tissue motions were detected or estimated, and the analysis was focused on soft tissue movements. Indeed, the analyzed movements were performed by mimic muscles, and only in *surprise with mouth open* the temporomandibular joint was moved by masticatory and supra-hyoid muscles.

In the current study, both symmetric and asymmetric facial movements were selected. Maximal movements (border movements) were used because they are more likely to enhance motion problems in patients (Weeden *et al.,* 2001). Each facial movement should be characteristically performed only in well defined parts of the face (Coulson *et al.,* 2002; Giovanoli et al, 2003; Trotman *et al.,* 1996). Three-dimensional facial movements were computed after

subtraction of all head and neck motions, using the three head markers to define a new reference system (Cappozzo *et al.,* 2005). This mathematical operation allowed the subjects to perform the facial animations freely, without any restriction to head motion (Trotman and Faraway, 1998; Trotman *et al.,* 1998b). In contrast, other protocols restricted head motion (Linstrom, 2002; Linstrom *et al.,* 2002). Subsequently, the three-dimensional vector of maximum displacement between rest position (the starting, reference position) and the maxima of the motion was computed, similarly to the Maximal Static Response Assay (MSRA) reported by Wachtman *et al.,* (2000). Further analyses will consider the actual path of motion of each marker, using the three-dimensional coordinates collected in each frame of motion. Indeed, most of previous studies analyzed only maximum movements (Trotman and Faraway, 1998), and did not report detailed, quantitative assessments of the paths of motion of single landmarks. In several instances (Frey *et al.,* 1999; Giovanoli *et al.,* 2003; Wachtman *et al.,* 2000) only examples of paths of motion were presented, but no statistical analysis performed. Quantitative assessments of the movements paths were made only by Tarantili *et al.* (2005), and by Trotman *et al.,* (2004).

To detect actual variations between and within individuals, the signal-to-noise ratio of each measuring system should be known Johnston *et al.,* (2003). The optoelectronic instrument used in the present study was calibrated with an accuracy lower than 0.02%. This means that the movement of each 2-mm marker could be detected within 0.12 mm. This high accuracy does not have an immediate practical, biological significance, unless the minimal motion threshold is known. Indeed, in a system that mixes facial expressions (which could be very variable) and a reduced working volume (the face, and in particular the mouth and the eyes), the assessment of repeatability is mandatory: only movements larger than the minimal *noise* level are of biological significance.

Therefore, a measurement protocol was devised, and intra-session and inter-session repeatability assessed in young, healthy volunteers. According to Johnston *et al.* (2003), reproducibility can be met when variations are lower than 1 mm. If this criterion is valid, in current study only the *surprise with closed mouth* in men met the standard for the intra-session variations. If less stringent thresholds are used Trotman *et al.,* (1998a), all our expressions could be considered reproducible (mean TEMs all lower than 3.3 mm). For the inter-session variations, all but the left eye closure had standard deviations lower than 1 mm.

Indeed, the current intra- and inter-session variability in single landmark movements was well comparable (or even better) to previous literature reports (Johnston *et al.,* 2003; Trotman *et al.,* 1996, 1998a; Weeden *et al.,* 2001). Also, the expression- and marker-related variations in repeatability were already reported: for instance, a larger repeatability in the maximum (instructed) smile than in the free smile movement was found by Johnston *et al.*, 2003. Trotman *et al.*, 1998a found that intra-session repeatability depended on marker and movement. Overall, lip landmarks appear to be the less reproducible Johnston *et al.,* (2003).

The present sex-related differences in the repeatability of facial movements (men more repeatable than women, except for maximum smile) cannot be directly compared to some literature reports where male and female data were pooled (Weeden *et al.,* 2001), and some contrasting results were found by Johnston *et al.*, 2003 who reported a similar repeatability in women and in men for almost all facial expressions. Interestingly, also Johnston *et al.*, 2003 found that women were more repeatable than men in the maximum smile. The differences in repeatability of the two asymmetric movements was unexpected, also considering that the mimic muscles of the upper part of the face receive bilateral nervous commands; a possible laterality in facial muscles may be hypothesized, as well as a different right-left training. These differences should be carefully investigated in futher studies.

The three-dimensional asymmetry indices had a limited repeatability both within- and between-sessions, a finding reported also by Trotman and Faraway (1998). Indeed, the use of indices with a large individual variability may have a limited practical application, but the definition of normal levels of asymmetry is mandatory for the analysis of patients with unilateral lesions. During the execution of symmetric movements, asymmetric motions of paired landmarks have been reported in normal persons by some investigators (Coulson *et al.,* 2002; Trotman *et al.,* 2000; Tzou *et al.,* 2005), but denied by others (Linstrom, 2002; Linstrom *et al.,* 2002). Overall, it appeared that nasal asymmetry

was very variable, and the location of these markers should be carefully controlled, and eventually their calculations skipped. Indeed, nasal markers are among those with the least reproducibility (Trotman *et al.,* 1996).

In conclusion, the protocol devised in the current study demonstrated a sufficient repeatability for a future clinical application. The use of both symmetric and asymmetric facial expression may allow a better definition of the impairments of patients with unilateral facial lesions. Great care should be taken to assure a consistent marker positioning in all the subjects. The next step would be the definition of reference values for three-dimensional facial movements in subjects of different ages and of both sexes.

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New findings on 3-D microanatomy of cellular structures in human tissues and organs. An HRSEM study

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We present here findings obtained on a large number of human tissues over a period of more than ten years, by our modification of the Osmium maceration method for high resolution scanning electron microscopy (HRSEM). Data are documented by original pictures which illustrate both some 3-D intracellular features not previously shown in human tissues, and results obtained in our current studies on mitochondrial morphology and on the secretory process of salivary glands.
We have demonstrated that mitochondria of cells of practically all human tissues and organs have usually tubular cristae, and that even the cristae that look lamellar are joined to the inner mitochondrial membrane by tubular connexions similar to the crista junctions later seen by electron tomography. Concerning salivary glands an important result is the development of a morphometric method that allows the quantitative evaluation of the secretory events.

Key words: HRSEM, maceration method, mitochondria, crista junctions, salivary glands, secretory process, cell organelles.

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The OsO_4 maceration method for high resolution scanning electron microscopy (HRSEM), introduced in the eighties by Tanaka and his co-workers (Tanaka, 1980; Tanaka and Naguro, 1981; and particularly that by Tanaka and Mitsushima, 1984), aroused great interest for its unique ability of providing three dimensional (3-D) images of intracellular membranaceous structures.

In a matter of a few years, however, the use of the technique declined. According to our experience, this is mainly due to the fact that the protocol is too long and rigid, and that the use of the freeze-cracking procedure for cutting the samples does not allow the analytical study of a whole specimen, as it is compulsory in the case of human needle biopsies. Thus, in order to make the technique more suitable for the study of human tissues, we embedded strips of fixed tissue samples, about 2 mm thick and 7 mm long in agarose, and cut them entirely into sections of 100-150 μm by using a tissue sectioner at room temperature (Riva *et al.*, 1993). Later on (Riva *et al.*, 1999), we introduced, as secondary fixative, the mixture 1% OsO_4 - 1.25% $K_4Fe(CN)_6$ that enhances osmium binding to tissue, thereby rendering unnecessary the long treatments with the binding agent tannic acid suggested by Tanaka and Mitsushima (1984). This modification not only reduced the preparation time from eight days to three days or fewer, but also eliminated a source of contamination and made the whole procedure more reproducible and easy to perform. Another advantage of our method was that, following the secondary fixation by the above mentioned mixture, the procedure can be suspended for several days by storing the specimens in phosphate buffered saline (PBS) at 4C°. Finally, by shaking the tissues during maceration by a rotating agitator, we succeeded in removing all cytoplasmic organelles, thus visualizing, for the first time, the cytoplasmic side of the plasmalemma and its specializations.

Materials and Methods

Though samples from tissues taken from experimental animals were occasionally studied, most of the findings reported here refer to specimens obtained from patients undergoing surgery for removal of tumors, and to needle biopsies. Informed consent was obtained from each patient and permission was granted by the local ethical committee (ASL 8, Cagliari). Normality of tissues of surgical origin was assessed by parallel examinations on the same tissues treated for light microscopy (LM). The protocol used was the following:

1. Fixation: strips of tissue of 1-2 mm x 7 mm were fixed with 0.5% glutaraldehyde + 0.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), 15 min at room temperature (RT)
2. Rinsing: PBS 3 x 10 min at RT
3. Postfixation: 1% OsO_4 - 1.25% $K_4Fe(CN)6$ in distilled H_2O, 2 h in the dark at 4°C
4. Rinsing: PBS 3 x 10 min at RT; specimens can be stored in this solution at 4°C for a maximum of 15 days
5. Sectioning: specimens are embedded in 1% agarose in distilled H_2O and cut into 150 μm thick sections by a TC2 Sorvall tissue sectioner at RT
6. Rinsing: PBS 3 x 10 min at RT
7. Second postfixation: 1%OsO_4-1.25% $K_4Fe(CN)6$ in distilled H_2O, 1 h in the dark at 4°C
8. Rinsing: PBS 3 x 10 min at RT
9. Maceration: 0.1% OsO_4 in PBS, 44-48 h at 25°C
10. Rinsing: PBS 3 x 10 min at RT
11. Dehydration through a graded acetone series, Critical Point Drying with CO_2, Coating with platinum (2 nm) by an Emitech 575 turbo sputtering apparatus
12. Observation by a FE HRSEM Hitachi S4000 operated at 15-20 kV

Since we have found that certain specimens, e.g. the testis, striated muscles, tissue culture cells, and pellets of isolated organelles were refractory to sectioning at RT, we rapidly froze them in liquid nitrogen, and then shattered by a blow of a hammer. The multiple salvaged small fragments were treated in precisely the same manner as are tissue slices. It must be noted, however, that when we performed both methods on the same tissues (*see below*), results concerning some organelles, such as mitochondria, were slightly different. Freeze cracking resulted, in fact, in a very sharp and regular plane of section transecting all organelles, whereas in specimens sectioned at RT, the exposed surfaces looked less regular and details more three dimensional. Moreover, in the latter specimens, owing to the irregularity of the plane of section, some organelles (i.e.: nuclei, mitochondria, etc.) were not transected, allowing the visualization of their whole 3-D configuration.

Results and Discussion

Although the osmium maceration has been originally introduced for LM more than one century ago (Bolles Lee and Henneguy, 1887), its mechanism of action is still partially known. The prevailing idea is that dilute osmium produces a progressive cleavage of cellular proteins (Maupin and Pollard, 1983; Behrman, 1984), preserving, to an extent, membranaceous structures.

In this report we describe a number of findings obtained by applying our osmium maceration technique to several hundreds of human specimens. The first set of illustrations shows some 3-D intracellular features not previously shown in human tissues, whereas the second one is devoted to structures more related to our current studies, which are mainly focused on the morphology of mitochondrial cristae and on the study of the secretory process of salivary glands.

As stated above, by shaking the specimens with a rotating agitator during maceration, cytoplasmic organelles may be partially or totally removed, leaving the cytoplasmic side of the plasmalemma available to inspection. This applies even to nuclei whose chromatin can be removed allowing the visualization of the inner side of the nuclear envelope and of its complement of nuclear pores (Figure 1). Organelles can be removed only partially, as demonstrated by Figure 2 that shows in a cell of a striated duct, by the cytoplasmic side, a portion of the apical membrane that is dotted by holes corresponding to the bases of microvilli, deprived of the cytoskeleton. In the same picture there are also some tubules of the smooth endoplasmic reticulum enveloping not-transected mitochondria that morphologically closely resemble bacteria. The inset of the same figure represents some cilia, whose sections clearly demonstrated their microtubular components. In a secretory cell of a major sublingual gland there are some annulate lamellae, which are in continuity with the cisternae of the rough endoplasmic reticulum that are covered by ribosomes (Figure 3). The fenestrations of the annulate lamel-

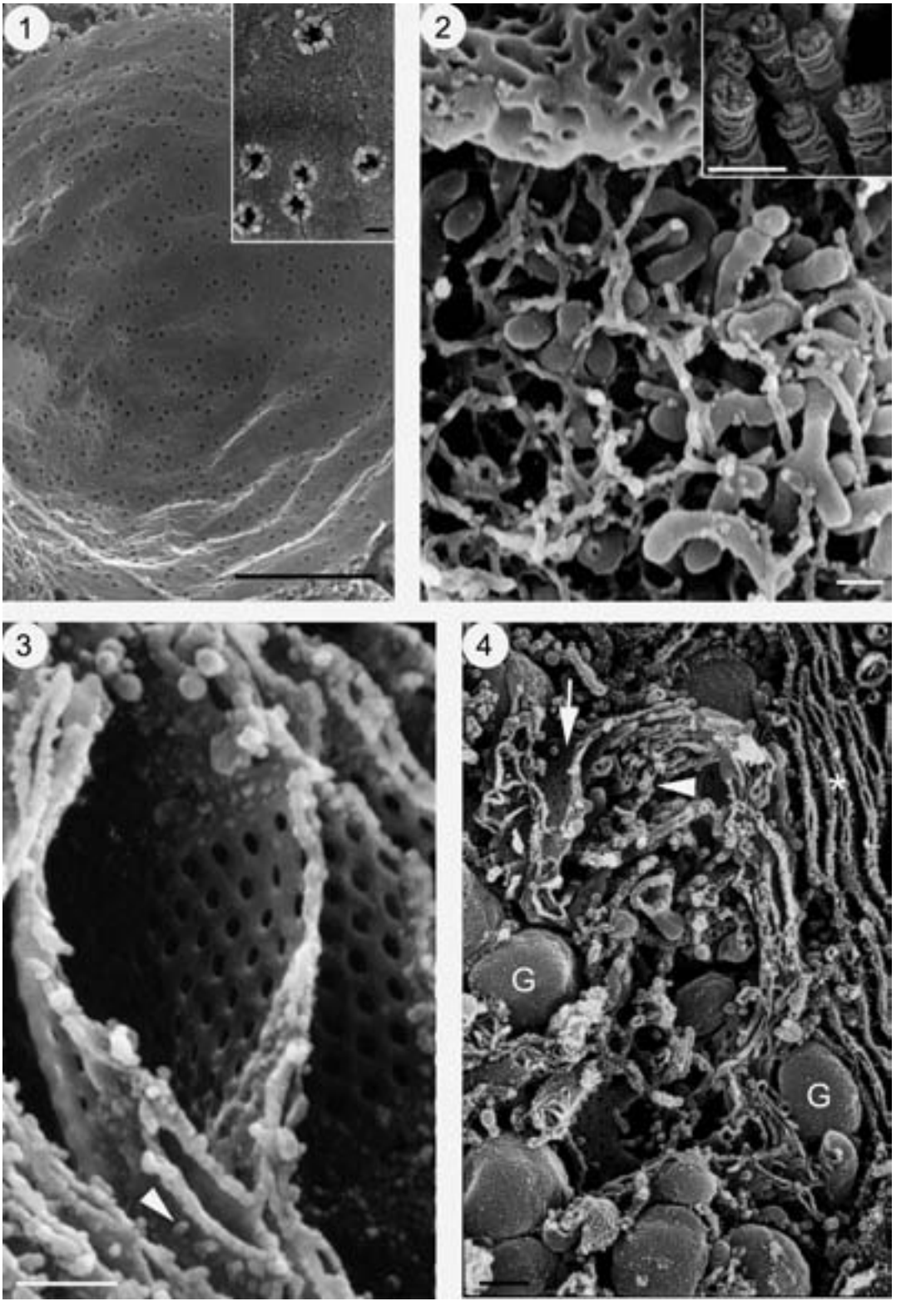

Figure 1. Internal surface of a nucleus deprived of its chromatin. Bar: 1.5 µm. The inset shows some pore complexes. Bar: 100 nm.
Figure 2. Cell of a striated duct. In the upper portion there is the cytoplasmic side of the luminal membrane, dotted by holes corresponding to the bases of apical microvilli. Below it there are many intact mitochondria enveloped by elements of the smooth endoplasmic reticulum. Bar: 1 µm. The inset shows a few macerated cilia from the mucosa of the human maxillary sinus. Bar: 500 nm.
Figure 3. Annulate lamellae in a cell of a human sublingual gland. The adjoining cisternae of the endoplasmic reticulum are covered by ribosomes (arrowhead). Bar: 500 nm.
Figure 4. Golgi apparatus of a serous cell. The cisternae exhibit a curved profile, budding vesicles (arrowhead), and numerous fenestrations (arrow). Secretory granules (G) and cisternae of the rough endoplasmic reticulum (asterisk) also are seen. Bar: 1 µm.

lae look more numerous and regularly arranged than those observed in the nuclear envelope and unlike the latter do not bear pore complexes (Figure 3). The Golgi complex (Figure 4) of a serous cell, clearly demonstrates its relationships with the elements of the rough endoplasmic reticulum and with the secretory granules. Also its cisternae exhibit many fenestrations and budding vesicles.

We become interested in the structure of mitochondrial cristae soon as we applied the osmium maceration method to human tissues. In our first report on the method, the one in which we introduced the sectioning at RT (Riva *et al.*, 1993) we published, in fact, pictures from human salivary glands, kidney and liver, showing both entire mitochondria and transected ones. The latter, confirming the pioneering findings obtained in rat mitochondria by Lea and Hollenberg (1989), were endowed with tubular cristae. Since then, we started an investigation on the 3-D features of mitochondria from a variety of organs that is still in progress.

From the beginning, our results by HRSEM matched those reported (Mannella *et al.*, 1994) following the reconstruction of the internal structure of mitochondria by high voltage transmission electron microscopy tomography (HVTEMT). We have shown, in fact, that mitochondria from a large variety of human and animal organs, have mostly tubular cristae (Figure 5, inset) and that lamellar cristae (Figure 5, inset) are joined to the inner mitochondrial membrane by tubular connexions. Such tubular connexions (Figure 5), that were not seen by Lea *et al.* (1994) who used the freezing cracking method, were documented by our technique since 1995 (Riva *et al.*, 1995a; Riva *et al.*, 1995b). They were named crista junctions using HVTEMT (Mannella *et al.*, 1997, Perkins *et al.*, 1997). It must be remarked, furthermore, that the latter technique, which requires laborious calculations, is performed on a very limited number (usually less than ten) of organelles (Perkins *et al.*, 1997; Prince and Buttle, 2004), and thus can hardly demonstrate structural differences between mitochondria of different organs, nor pathological variations that were, instead, clearly shown in our specimens (Faa *et al.*, 1997; Riva and Tandler, 2000; Ambu *et al.*, 2000). On the other hand, in Figure 6, that shows, side by side, an oxyphil and a chief cell of a human parathyroid gland in a preparation obtained by freeze-cracking, we can observe the structural diversity between their relevant mitochondria. Moreover, by comparing the oxyphil cell mitochondria shown in Figure 6 with those of a similar cell sectioned at RT (Figure 5), it clearly emerges that our technique gives a far better 3-D and detailed view of the cristal morphology. Another finding (Figure 7) first reported by us (Riva *et al.*, 2003), thanks to our maceration method, is the fact that mitochondria of steroid producing cells have moniliform cristae with bulbous tips. These tips gave in thin sections in transmission electron microscopy (TEM), the impression, reported in textbooks (e.g. Bloom and Fawcett, 1994), that mitochondria of such type of cells have tubular and vesicular cristae. Recently, (Riva *et al.*, 2005; Riva *et al.*, 2006) we have successfully applied our technique to investigate structural differences in two biochemically defined populations of isolated rat cardiac mitochondria, and to study the structure of cristae in relation to aging.

As can be seen from Figure 8 we have been able to remove all cytoplasmic organelles of serous cells of salivary glands in order to expose the cytoplasmic side of the intercellular canaliculi. In the same preparations we demonstrated regular clusters of particles that we related to cellular junctions (Testa Riva *et al.*, 2003). We took advantage of having a view of a relatively large area of the membrane of the canaliculi, the site where exocytosis occurs, in order to investigate, with morphometric methods, the dynamics of salivary secretion at the cellular level. We documented, by HRSEM (Testa Riva *et al.*, 2006), the changes of the portions of membrane involved into secretion after stimulation with secretagogue drugs. We set up an *in vitro* stimulation method of 1 mm^3 pieces of human normal glands which were incubated with various drugs for 30 min in oxygenated inorganic media (Riva *et al.*, 2002). To quantify the secretory response and to compare the activity of a given stimulant, we calculated, on HRSEM images, the number of the holes corresponding to microvilli and that of the microbuds (corresponding to TEM pits) seen on the cytoplasmic side of the canaliculi. In fact, as we have previously indicated on the basis of subjective observations (Segawa *et al.*, 1998), following secretory stimulation, microbuds increased, whereas microvilli were greatly reduced. Results of other experiments that dealed, both in submandibular and parotid glands, with the action of specific inhibitors are now under evaluation. It must be noted that our protocol based on the quantitative evaluation of 3-

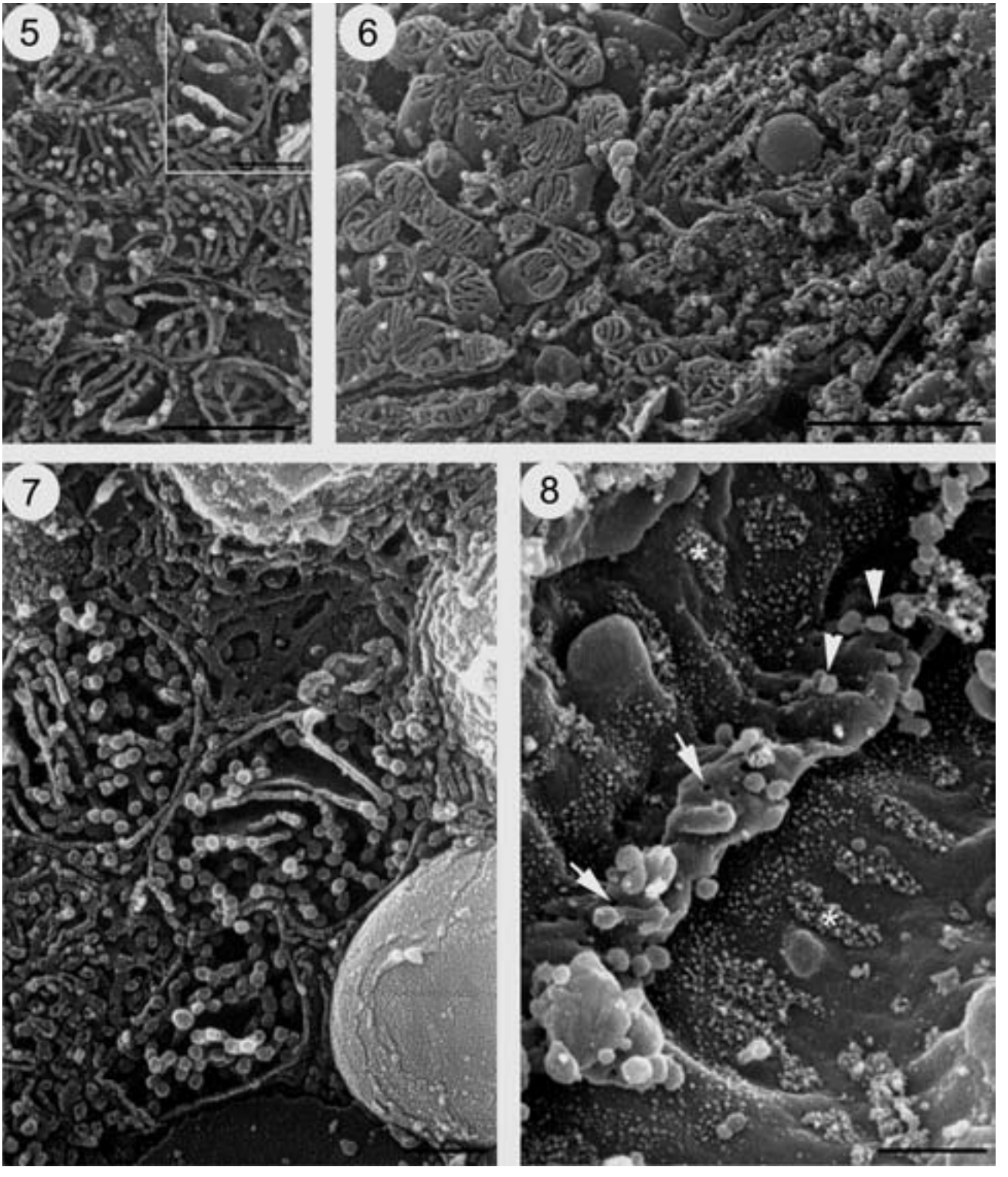

Figure 5. RT sectioned mitochondria of an oxyphilic cell of the human parathyroid gland exhibiting tubular cristae. Bar: 1 µm. The inset demonstrates a lamellar crista viewed en face and linked to the inner mitochondrial membrane by tubular connexions (crista junctions). Bar: 500 nm. Figure 6. Picture of an oxyphilic cell of the human parathyroid (left) and of an adjoining chief cell (right) obtained by freeze cracking the specimen. Note that mitochondria cristae look less 3-D than in the previous image (Figure 5) from an homologous cell sectioned at RT. Bar: 1.5 µm. Figure 7. Cristae with bulbous tips and moniliform constrictions are seen in mitochondria of a steroid producing organ (human adrenocortical gland, reticulate zone). Bar: 500 nm. Figure 8. Cytoplasmic side of the plasmalemma of a serous cell following removal of cytoplasmic organelles. The intercellular canaliculus exhibits microbuds (arrowheads), and holes (arrows) corresponding to the bases of microvilli. The continuous band and clusters of particles (asterisks) placed alongside it are related to junctional complexes. Bar: 500 nm.

D events induced by exocytosis on large fields obtained by HRSEM is, by far, the most reliable and easy to perform morphometric method to evaluate the secretory response. It avoids, in fact, the need of producing the large number of serial sections and the complex calculations required for TEM stereological procedures.

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Non-traditional large neurons in the granular layer of the cerebellar cortex

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The granular layer of the cerebellar cortex is composed of two groups of neurons, the granule neurons and the so-called large neurons. These latter include the neuron of Golgi and a number of other, lesser known neuron types, generically indicated as *non-traditional* large neurons. In the last few years, owing to the development of improved histological and histochemical techniques for studying morphological and chemical features of these neurons, some non-traditional large neurons have been morphologically well characterized, namely the neuron of Lugaro, the synarmotic neuron, the unipolar brush neuron, the candelabrum neuron and the perivascular neuron. Some types of non-traditional large neurons may be involved in the modulation of cortical *intrinsic* circuits, establishing connections among neurons distributed throughout the cortex, and acting as inhibitory interneurons (i.e., Lugaro and candelabrum neurons) or as excitatory ones (i.e., unipolar brush neuron). On the other hand, the synarmotic neuron could be involved in *extrinsic* circuits, projecting to deep cerebellar nuclei or to another cortex regions in the same or in a different folium. Finally, the perivascular neuron may intervene in the *intrinsic* regulation of the cortex microcirculation.

Key words: granular layer; non-traditional neurons; neuron of Lugaro; synarmotic neuron; unipolar brush neuron; candelabrum neuron; perivascular neuron.

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The granular layer (GL) of the cerebellar cortex shows a homogeneous structure in the different lobes, lobules, laminae and folia of the mammalian cerebellum, except for its depth, which varies in the different folia ranging from 400-500 μm, in the apical region of the folium, to about 100 μm, in the basal one. The GL is composed of two main groups of neurons: the granule neurons (*granules*) and the so-called large neurons (*non-granules*). The granules have a small, spheroid body, measuring 5-8 μm in diameter, and their bodies are packed within the GL at a density of 2 to 7×10^6 per mm^3. In comparison with the granules, the large neurons show a more voluminous body, with a diameter ranging from 15 to 25 μm, and a much lesser density. They include the neuron of Golgi, one of the five *traditional* corticocerebellar neurons (together with stellate, basket, Purkinje and granule neurons), and a number of other neuron types, generically indicated as *non-traditional* (n-trad) large neurons (Jansen and Brodal, 1958; Eccles *et al.*, 1967; Voogd and Glickstein, 1998; Mugnaini, 2000; Geurts *et al.*, 2003; Houck and Mugnaini, 2003; Flace *et al.*, 2004 Ito, 2006). Although the n-trad large neurons were first observed in the mammalian GL a long time ago and increasingly detailed descriptions have been provided over the years with a certain regularity (see, e.g., Golgi, 1874; Lugaro, 1894; Ramon y Cajal, 1911; Pensa, 1931; Landau, 1933; Fox, 1959), it has long been debated whether they should be considered as distinct neuron types or not. This has depended on objective difficulties existing in the observation and recognition of these neurons: (a) the Golgi silver impregnation technique constantly reveals only a small percentage of neurons (1-2%) and thus only rarely visualizes n-trad large neurons; (b) the large neurons are often literally hidden by granules, so the n-trad ones have in many cases been misinterpreted as traditional Golgi neurons; (c) finally, the n-trad large neurons may display differences in their distribution in the GL

and also in their shape and size. In recent years, owing to the development of improved histological and histochemical techniques for the study of nervous tissues, morphological and neurochemical parameters have been better defined allowing the discrimination between different n-trad large neuron types. A first classification of the n-trad large neurons of the human GL was made by Braak and Braak (1983). Using a technique that stains cytoplasmic deposits of lipofuscin, these Authors recognized 3 types of large neurons. In particular, Braak and Braak's types 2 and 3 comprise n-trad large neuron types, but in reality both include various subtypes, each having a different localization within the layer, different features of the bodies and processes and most probably different functions. More recently, an in-depth study of a great number of large neurons of the human cerebellar cortex, revealed by immunocytochemistry for glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme, has supplied a demonstration of various types of putative GABAergic n-trad large neurons (Flace *et al.*, 2004). Ambrosi and coll. have proposed a classification of these GAD-positive n-trad large neurons by reference to their localization and position in the GL (conventionally subdivided into three zones: external, intermediate and internal) and to the morphological features of their bodies and processes (for details, see Flace *et al.*, 2004). Up to now, five types of n-trad large neurons have been sufficiently characterized from the morphological and neurochemical standpoints, even if their functional roles are not yet completely understood. They include the neuron of Lugaro, the synarmotic neuron, the unipolar brush neuron, the candelabrum neuron and the perivascular neuron (Table 1).

Neuron of Lugaro

The neuron of Lugaro (NL; also known as intermediate neuron or horizontal neuron) Figure 1 has been described in the GL of various species of mammals, including humans (Lugaro, 1894; Fox, 1959; Braak and Braak, 1983; Lainé and Axelrad, 1996; Geurts *et al.*, 2001; Flace *et al.*, 2004; Melik-Musian and Fanardzhyan, 2004). The body of the NL is distributed in all cerebellar lobes and lobules, localized in the external zone of the GL just beneath the Purkinje neuron layer. The body is fusiform, horizontal (i.e., parallel to the folium surface), with a major axis measuring 15-25 μm and lying on the sagittal plane (i.e., orthogonal to the folium major axis). From the opposite body poles, two dendrite trunks originate, being sagittally oriented and running horizontally along the boundary between the GL and Purkinje neuron layer; they are rectilinear, remarkably long (up to 1 mm) and branch within strips of cortex ranging from the internal zone of the molecular layer to the external zone of the GL. The dendrites and body of NL offer a very extensive receptive surface, receiving most inputs from recurrent branches of Purkinje neuron axons and in addition from granule and basket neuron axons. The axon of the NL originates from one body pole, or from one dendrite trunk, and spreads with its collaterals in latero-lateral direction (i.e., parallel to the folium major axis) within the molecular layer. Axon terminals mainly form synapses upon basket and stellate neurons and apical dendrites of Golgi neurons.

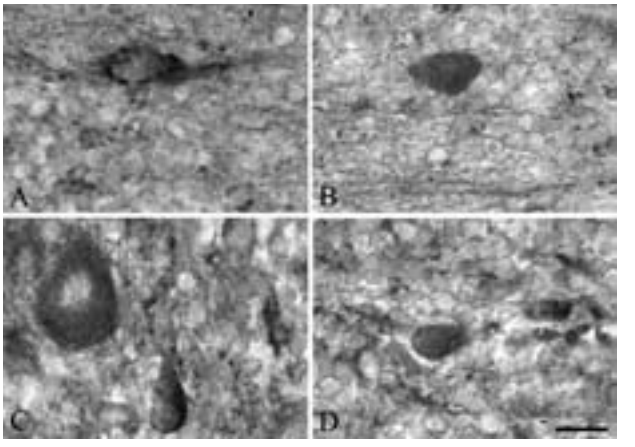

Figure 1. Non-traditional large neurons of the granular layer immunostained for glutamic acid decarboxylase. A. neuron of Lugaro; B. synarmotic neuron; C. candelabrum neuron; D. perivascular neurons. Scale bar: 20 μm.

A second type of NL has also been described, having a roundish or triangular body localized in the GL intermediate zone and with a different spatial process arrangement, but establishing synaptic contacts similar to those of the fusiform type (Lainé and Axelrad, 2002; Melik-Musian and Fanardzhyan, 2004).

Immunocytochemical investigations have demonstrated GAD or GABA immunoreactivity in the NL of the rat (Aoki *et al.*, 1986; Lainé and Axelrad, 1998) and human (Flace *et al.*, 2004), indicating its putative GABAergic, inhibitory nature, and also the presence of the inhibitory amino acid glycine and of a co-localization of glycine and GABA in the rat (Dumoulin *et al.*, 2001) and macaca monkey (Crook *et al.*, 2006). Other markers of the NL are

Table 1. Morphological features and classifications of *non-traditional* large neurons of the granular layer.

NEURON TYPE	*BODY LOCALIZATION with reference to: (a) Cerebellar Lobes; (b) Foliar Regions; (c) GL Zones*	*BODY FORM AND ORIENTATION*	*PROCESS FORM AND ORIENTATION*	*CLASSIFICATION PROPOSED BY BRAAK & BRAAK (1983)*	*CLASSIFICATION PROPOSED BY AMBROSI AND COLL. (Flace et al., 2004)*
Lugaro	a) All Lobes b) All Regions c) External Zone	Fusiform, Horizontal	Dendrites originate from opposite body poles and run horizontally at the boundary with PNL; axon spreads within the ML	Type 2	External Zone/ Type 3
Synarmotic	a) All Lobes b) Basal Region c) Internal Zone	Ovoid, Horizontal	Dendrites are confined in the GL; axon runs horizontally at the boundary with or inside the white matter	Type 2	Internal Zone/Type 2
Unipolar Brush	a) Flocculo-nodular Lobe b) All Regions c) All Zones	Roundish/ Ovoid, Vertical	Dendrite trunk originates from external body pole and spreads in the GL and ML; axon ramifies in the GL	Type 3	*Not visualized*
Candelabrum	a) All Lobes b) All Regions c) External Zone	Pear-shaped, Vertical	Dendrite and axon ascend from the external body pole to the ML	Type 3	External Zone/ Type 1
Perivascular	a) All Lobes b) All Regions c) All Zones	Roundish/Ovoid, variously oriented, perivascular	Processes: variously oriented, perivascular	*Not mentioned*	Perivascular Type

ML: Molecular Layer; PNL: Purkinje Neuron Layer; GL: Granular Layer.

the cytoplasmic antigen rat-303 (Sahin and Hockfield, 1990) and the calcium binding protein calretinin (Geurts *et al.*, 2001).

The NL, expanding in extensive, horizontally developed regions of cerebellar cortex, creates the anatomical conditions for the interconnection of many neurons, located in all cortex layers. It mainly receives inputs from Purkinje neurons and projects to: (a) stellate and basket neurons, which in turn project back to Purkinje neurons, which are the main source of outputs from the cortex; (b) Golgi neurons, which modulate the activity of afferent mossy fibres. Since Purkinje, Lugaro, stellate, basket and Golgi neurons are all GABAergic, inhibitory neurons (Benagiano *et al.*, 2001; Flace *et al.*, 2004), multiple *intrinsic* (i.e., non-projective) circuits, each formed by series of GABAergic synapses, exist in the cerebellar cortex, able to produce disinhibition (i.e., inhibition of an inhibition) phenomena which spread within extensive regions of the folium.

Synarmotic neuron

The synarmotic neuron (SyN; also known as neuron of Landau) Figure 1 was described by Landau (1933) in various mammals, including humans. This n-trad large neuron type has long been neglected, being occasionally cited in literature (see Jansen and Brodal, 1958), but only recently taken into consideration once more (Katsetos *et al.*, 1993; Flace *et al.*, 2004). The SyN is distributed in all lobes and lobules with a preferential localization in the basal and intermediate regions of the folium. The body, localized in the internal zone of the GL or, sometimes, in the subcortical white matter, is ovoid, horizontal, with a major diameter measuring 20-25 μm. The dendritic tree is confined in the GL, where it probably receives afferences from mossy fibres. The axon arises from a body pole and runs in the white matter, intermingled among efferent axons from, or afferent axons to, the cerebellar cortex. It finally re-enters the cortex, associating two cortical regions in the same folium or in different folia, or projects to cerebellar nuclei. A similar neuron was described by Braak and Braak (1983) and included in type 2 of their classification, but these Authors did not mention the SyN.

Little is known about the neurochemical features of the SyN. Immunoreactivity to GAD, suggesting a GABAergic nature (Flace *et al.*, 2004), and to the calcium binding protein calbindin (Katsetos *et al.*, 1993) has been detected.

The SyN is thought to be involved in extrinsic nervous circuits. It is a candidate for a second source (besides the Purkinje neuron) (see also Braak and Braak, 1983; Müller, 1994) of outputs from the cerebellar cortex, making associative cortico-cortical connections or projective connections onto deep cerebellar nuclei. This latter hypothesized role of the SyN, similar to that of the Purkinje neu-

ron, is in agreement with their common GABAergic nature.

Unipolar brush neuron

The unipolar brush neuron (UBN; also indicated as pale or monodendritic neuron) has been described principally in the GL of the flocculonodular lobe using histological and immunocytochemical techniques (Altman and Bayer, 1977; Braak and Braak, 1983; Hockfield, 1987; Munoz, 1990; Braak and Braak, 1993; Mugnaini and Floris, 1994; Dino *et al.*, 2000; Dogue *et al.*, 2005; Kalinichenko and Okhotin, 2005). The UBN has a roundish, or ovoid and vertical, body measuring 9-15 μm (thus intermediate in size between granules and other large neuron types), localized throughout the GL. From the external body pole a single dendrite trunk originates and gives rise at its apex to packed small branches, spreading in the GL and up to the neighbouring molecular layer and receiving synaptic contacts mainly from terminals of mossy fibres and axons of Golgi neurons. The axon ramifies in the GL and its branches end upon dendrites of granules, participating, like the terminals of mossy fibres, in the formation of glomerular synaptic complexes (intrinsic mossy fibres).

Research carried out on the mouse cerebellar cortex has indicated that the UBN is an excitatory cell, using glutamate as neurotransmitter (Nunzi *et al.*, 2001). It also expresses receptors for glutamate (Jaarsma *et al.*, 1998; Geurts *et al.*, 2001), immunoreactivity to rat-302 antigen (Hockfield, 1987), chromogranin A (Munoz, 1990) and calretinin (Braak and Braak, 1993; Floris *et al.*, 1994; Geurts *et al.*, 2001).

The UBN mainly receives excitatory, glutamatergic synapses from mossy fibres and in turn projects its excitatory, glutamatergic axon on granules, which are also excitatory, glutamatergic. In this way, a powerful feed-forward amplification system of excitatory signals is created, coming from outside the cerebellum and reaching, via parallel fibres, the dendritic trees of projective Purkinje neurons.

Candelabrum neuron

The candelabrum neuron (CN; also known as intercalated neuron) Figure 1 has been described ubiquitously in the cerebellar cortex of the rat (Lainé and Axelrad, 1994). It has a pear-shaped body, with a vertical (i.e., orthogonal to the surface) major axis, measuring 20-25 μm. Its body is squeezed against the internal body pole of a Purkinje neuron or, together with others, forms a row that joins up with that of Purkinje neuron bodies. From the external body pole, dendrite trunks originate, that ascend through the molecular layer and arborize there in a candelabrum-like fashion, as well as a thin axon that also spreads in the molecular layer. Moreover, basal dendrites originate from the internal body pole and ramify in the GL. The CN receives inputs from axon recurrent collaterals of Purkinje neurons and from granule and basket neuron axons. Its axon forms synapses upon basket and stellate neurons, but not upon Purkinje neuron dendrites.

Although the CN has only recently been described, a neuron type with similar morphological features had already been observed in the cat cerebellar cortex by Pensa (1931). Moreover, this neuron type had also been visualized by Braak and Braak (1983) and included in type 3 of their classification.

Recently, Flace *et al.* (2004) demonstrated that the CN shows immunoreactivity to GAD, also reporting that this is the most frequent GAD-immunoreactive large neuron type in the GL of the human cerebellar cortex. Preliminary data have indicated the presence of calbindin immunoreactivity within the CN (Flace *et al.*, unpublished datum).

Owing to its inhibiting, GABAergic nature and in view of the vertical displacement of its processes, the CN may play the role of inhibitory interneuron provided with intrinsic connections and mainly involved in modulation of the activity of inhibiting, GABAergic stellate and basket neurons (see also Lainé and Axelrad, 1998).

Perivascular neuron

The perivascular neuron (PN) Figure 1 is an n-trad large neuron type found in all cerebellar lobes and lobules and in all GL zones (Flace *et al.* 2004). It displays an isodiametric body, lying extensively along the wall of intracortical capillaries. Its processes also run for tracts of various length in a close anatomical relationship with capillaries.

GAD immunoreactivity has been observed in the body and processes of the PN (Flace *et al.* 2004).

In accordance with a supposed role of GABA in the local nervous regulation of intrinsic microvessels of the cerebellar cortex (Benagiano *et al.*, 2001), a vasoregulatory function has been hypothesized for the PN, possibly in part exerted by *volume transmission mechanisms* (Flace *et al.*, 2004).

Differential diagnosis

As previously noted, the n-trad large neurons of the GL are often difficult to recognize. Immunocytochemical techniques have made major contributions to their more precise identification.

The NL and CN, as well as the *traditional* large neuron of Golgi, are all localized in the external zone of the GL and display immunoreactivity for GAD or other GABA-related markers (Benagiano *et al.*, 2001; Flace *et al.*, 2004). The NL, like the neuron of Golgi, is immunoreactive to the cytoplasmic antigen rat-303 (Sahin and Hockfield, 1990), but, unlike the Golgi, it is positive for calretinin and negative for the glutamate receptor mGlu-R2 (Geurts *et al.*, 2001). On the other hand, the CN is immunonegative for rat-303, calretinin and mGlu-R2 (Sahin and Hockfield, 1990; Geurts *et al.*, 2001), but positive for calbindin (Flace *et al.*, *unpublished data*).

The UBN differs from the Golgi neuron and the LN and CN, apart from its topography, also because it never displays positivity for GABA-related markers (or for other inhibitory neurotransmitters) (Floris *at al.,* 1994), in accordance with its glutamatergic, excitatory nature (Nunzi *et al.*, 2001). Like the NL, but unlike the Golgi, the UBN shows positivity for calretinin (Geurts *et al.*, 2001); like the Golgi, but unlike the NL, it expresses mGlu-R2 (Geurts *et al.*, 2001); unlike both the NL and Golgi, it is negative for rat-303 (Sahin and Hockfield, 1990), but positive for rat-302 antigen (Hockfield, 1987).

The SyN, like the Golgi, NL and CN, displays positivity for GABA-related antigens (Flace *et al.,* 2004), but, unlike the other three large neuron types, it is internally localized in the GL and thus easy to discriminate. It also expresses positivity for calbindin (Katsetos *et al.*, 1993).

Some conclusive remarks

Although new data are rapidly accumulating, the knowledge of the n-trad large neurons of the GL is still incomplete and concerns only some neuron types. However, a number of functional roles could be attributed to the n-trad neurons.

(1) They may be involved in complex intrinsic circuits of the cerebellar cortex, some establishing horizontal (i.e., the NL) and some vertical (i.e., the CN) connections among neurons distributed throughout the cortex, acting as inhibitory (i.e., the NL, the CN) or excitatory (i.e., the UBN) interneurons. N-trad large neurons may thus modulate: signal transmission from afferent fibres to the cortex; the activity of granules, responsible for transduction of mossy fibre signals onto Purkinje neurons (granule-Purkinje neuron pathway); the activity of Purkinje neurons, main source of outputs from the cortex.

(2) A type of n-trad large neuron, namely the SyN, could be involved in extrinsic circuits of the cortex associating different regions of the cortex or projecting to cerebellar nuclei.

(3) The PN could represent a type of n-trad large neurons intervening in the local regulation of blood microcirculation.

A better knowledge of all these n-trad large neuron types will probably provide a decisive contribution to the task of unravelling the complex mechanisms on which the working of the *cerebellar neuronal machine* is based.

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The solitary chemosensory cells and the diffuse chemosensory system of the airway

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Solitary chemosensory cells (SCCs), which resemble taste bud cells, are present in the epidermis and oropharynx of most primary aquatic vertebrates. Recent studies have led to the description of SCCs also in mammals too. In the airway and digestive apparatus, these elements form a diffuse chemosensory system. SCCs do not aggregate into groups and in SCCs, as in taste bud cells, immunoreactivity for the G-protein subunit α-gustducin and for other molecules of the chemoreceptive cascade was found. Questions remain about the role of the diffuse chemosensory system in control of complex functions (e.g. airway surface liquid secretion) and about the involvement of chemoreceptors in respiratory diseases. Therapeutic actions targeting chemoreceptors could be tested in the treatment of respiratory diseases.

Key words: taste, chemoreceptor, gustducin, quorum sensing, trachea.

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The concept of a diffuse chemosensory system (DCS) has been defined in the last ten years and has rapidly changed the anatomical description of the respiratory and digestive apparatuses.

In some parts of these apparatuses, unexpected chemoreceptorial capabilities seem to be linked to the presence of a differentiated system of sensory elements, which appear related to the gustatory cells forming the taste buds of the oropharyngeal cavity. These elements are called solitary chemosensory (or chemoreceptor) cells (SCCs), and display analogies with homologous elements described in lower vertebrates. The possible functional roles of this cell system are open to discussion.

Solitary chemosensory cells

In the past, SCCs were considered typical of aquatic vertebrates. The absence of descriptions of these elements in terrestrial vertebrates led to the hypothesis that they disappeared with the aquatic-terrestrial transformation of vertebrates. As an example, in fish the skin and the oropharyngeal surfaces are provided with chemoreceptors, not organized into end organs, related to the gustatory system (Whitear, 1992). Similar elements are also located at the gills. The chemical information provided by these chemoreceptors is used for feeding or to detect predators (Peters *et al.*, 1991; Finger, 1997). In amphibians, the presence of cells with the morphological features of SCCs was described in the oral cavity of *Rana esculenta* (Osculati and Sbarbati, 1995).

A series of studies has led to the description of SCCs in mammals analogous to similar systems present in aquatic vertebrates (Sbarbati *et al.*, 1998; Sbarbati and Osculati, 2003). The first descriptions were obtained in the oral cavity, where SCCs recognizable on the basis of ultrastructural morphology and the presence of the taste cell-related G-protein subunit α-gustducin are present in the vallate papillae of the rat tongue during the first

days of extrauterine life (Sbarbati *et al.*, 1998 and 1999). Similar elements are also present in the palate of rodents (El Sharaby *et al.*, 2001). Further investigation led to the description of similar elements in large parts of the digestive and respiratory apparatuses.

SCC phenotype

SCCs form a rather polymorphic population, although some characteristics seem to be common to a majority of them. In general, they are slender epithelial elements, which display cytological characteristics suggesting a chemosensory role and which possess signalling mechanisms typical of taste cells (Sbarbati *et al.*, 1998, 2004a; Finger *et al.*, 2003). Often they are single, bipolar epithelial cells contacted by nerves and lacking a specialized connective bed (Figure 1). SCCs may be surrounded by glial-like epithelial cells.

In fish, common ultrastructural features of SCCs include spindle shape, basal synapses, abundant endoplasmic reticulum within the proximal part of the cell, and an apical microvillus. The distal processes of SCCs contain a distinct Golgi apparatus and characteristic vesicles (Whitear and Kotrschal, 1988). Where the epidermis is thick, the nucleus of the sensory cell often lies at the level of the second tier of epithelial cells from the surface, but in other situations the cell may be elongated, with its deep pole immediately above the basal layer of the epidermis. Usually, the apical process is of sufficient length to raise the presumed receptive membrane above the mucus covering the surface of the epithelium. Within the non-olfactory nasal epithelium of mammals, SCCs are morphologically similar to the individual cells in taste buds, but unlike taste cells, they form distinct synapses on cutaneous nerve fibers of the trigeminal nerve (Finger *et al.*, 2003).

In aquatic vertebrates, electrophysiological recordings supported the hypothesis that SCCs are chemosensory (Peters *et al.*, 1991) and that they respond to predator-avoidance or food-related stimuli, although they do not respond to some typical taste stimuli (Silver and Finger, 1984).

The molecular mechanisms of taste transduction

The detection of chemoreceptorial elements in apparatuses of endodermic origin has mainly been due to enormous developments in our knowledge of gustatory science. These developments led to a detailed description of the molecular machinery responsible for taste transduction. Five taste qualities exist (i.e., sodium salt, acids, amino acids, sweet and bitter) (Lindemann, 2001; Margolskee, 2002; Perez *et al.*, 2003). All taste pathways converge on common elements that mediate a rise in intracellular Ca^{2+} followed by transmitter release. Taste responses to bitter/sweet compounds and amino acids are initiated by G-protein-coupled receptors (GPCRs) and transduced via G-protein signalling cascades (Chaudhari and Roper, 1998; Gilbertson *et al.*, 2000).

In taste cells GPCRs are implicated in taste signal transduction (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000; Chaudhari *et al.*, 2000; Max *et al.*, 2001; Nelson *et al.*, 2001, 2002; Li *et al.*, 2002; Amrein and Bray, 2003). Differences between taste qualities are linked to different families of these receptors expressed in sets of taste receptor cells (Adler *et al.*, 2000; Nelson *et al.*, 2001; Zhang *et al.*, 2003). Bitter compounds activate bitter taste T2R/Trb receptors, which are encoded by a separate gene family consisting of about 30 members in mice. T2R receptors then activate gustducin heterotrimers. Activated alpha-gustducin stimulates phosphodiesterases to hydrolyze cAMP; the decrease in cAMP levels may modulate cyclic nucleotide regulated ion channels and/or kinases. Beta and gamma subunits of gustducin activate phospholipase C of the β2 subtype to generate IP3, which leads to release of Ca^{2+} from internal stores via activation of inositol 1,4,5-triphosphate receptor type III (IP3R3). Detection of amino acid and sweet compounds is mainly effected by the Tas1R (or T1R) gene family, which encodes three conserved receptors that function as heterodimers and form either a sugar receptor (Tas1R2/Tas1R3) or a general amino acid receptor (Tas1R1/Tas1R3). More in detail, the candidate receptors for amino acid taste transduction are ionotropic glutamate receptors, metabotropic glutamate receptors and in particular tastemGluR4, which is a truncated form of the brain mGluR4, lacking most of the N-terminal extracellular domain as well as the Tas1R1–Tas1R3 heteromer (Chaudhari *et al.*, 2000; Li *et al.*, 2002; Nelson *et al.*, 2002; Ruiz *et al.*, 2003; He *et al.*, 2004). Tas1R1 and Tas1R3 are coexpressed in taste buds in the anterior part of the tongue (Nelson *et al.*, 2001), while taste mGluR4 is expressed in taste buds of the circumvallate and foliate papillae (Yang

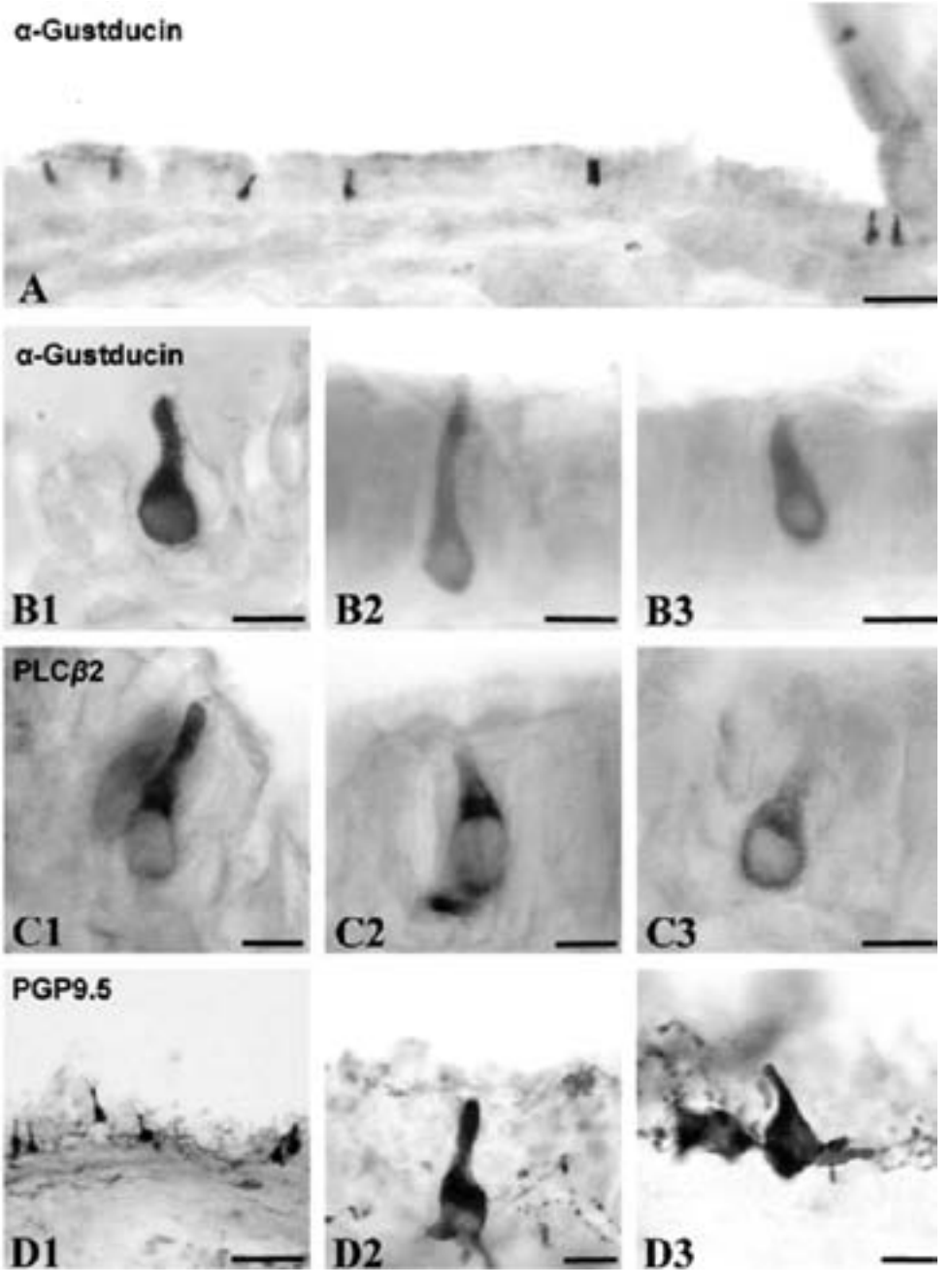

Figure 1. Staining pattern in the specific laryngeal sensory epithelium by α-gustducin (A, B1–B3), or PLC β2; C1–C3), and protein gene product (PGP) 9.5 (D1–D3) immunocytochemistry. Light microscopy images were obtained from free-floating sections that were subsequently observed by electron microscopy. Scale bars: 50 µm in A; 5 µm in B1,B3, C3; 10 µm in B2; 2.5 µm in C1,C2, D2,D3; 15 µm in D1. From Sbarbati *et al.*, 2004.

et al., 1999). Tas1R2–Tas1R3 is a GPCR activated by most known sweeteners (Nelson *et al.*, 2001).

The histochemical markers of the chemoreceptorial molecular cascade

Olfactory receptor neurons, taste cells and SCCs both utilize signal transduction cascades involving different G-proteins. A marker that has been largely used for morphological detection of chemosensory elements is gustducin. Gustducin is a heterotrimeric guanine-nucleotide binding protein (G protein), the existence of which was demonstrated in rats (Mc Laughin *et al.*, 1992) and then confirmed in man (Takami *et al.*, 1994). Although in the original studies gustducin was considered to be specific to a subset of taste cells, immunoreactivity for α-gustducin was later found in the brush cells of the digestive apparatus (Hofer and Drenckhahn, 1996; Hofer *et al.*, 1996,1999), in SCCs and in the vomeronasal organ. Thus, several studies have demonstrated that gustducin is a marker of chemosensitive cells.

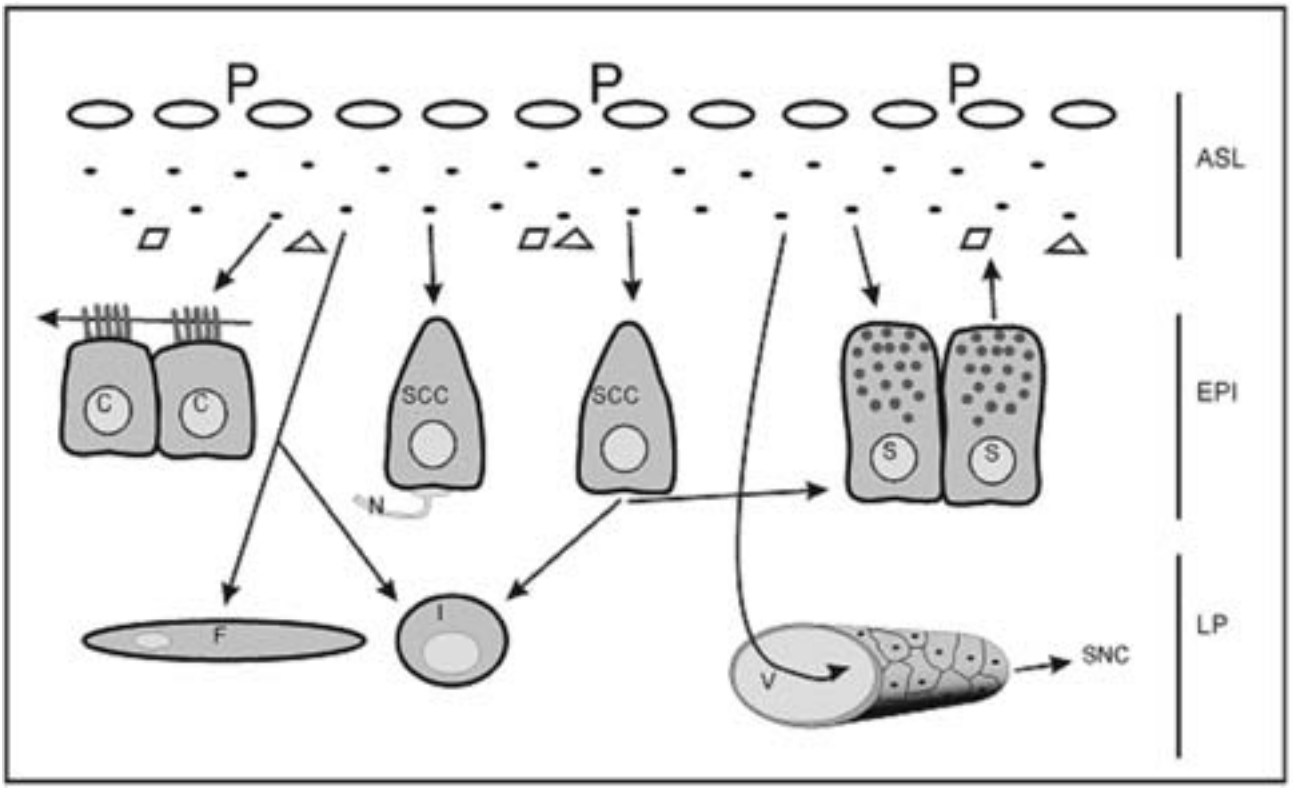

Figure 2. Schematic draft of lines of defense in the mammalian airway against AIs (dots) secreted by prokaryotes (P). A *first defensive line* is in the ASL, where binding proteins (triangles) or surfactant-like material (squares) are present. The *second defensive line* is in the epithelium (EPI), where AIs can interact with ciliate (C), secretory (S) or chemosensory cells (SCC). The presence of intraepithelial lymphocytes has not been taken into consideration. Innervated SCCs are contacted by afferent axons (N). Non-innervated, paracrine SCCs are also present. A possible secretory role for a sub-family of SCCs has been hypothesized. The *third defensive line* is located in the lamina propria (LP), which AIs can reach through interruptions in the epithelial layer. AIs act on fibroblasts (F), immune elements (I) or globule leukocytes. A *fourth defensive line* is linked to a probable systemic diffusion of AIs by vessels (V). In principle, AIs could cross the blood-brain barrier and their possible passage could be important in the "sickness behavior" described in parasitic diseases.

Apart from gustducin, several other molecules can be used to detect SCCs. A first approach is detection of membrane receptors. Taste cells express seven specific transmembrane G-protein coupled receptors. Different names are used to indicate these molecules in various classes of vertebrates. In rodents, T1R and T2R are generally recognized. Both these classes of receptors are linked to gustatory chemosensitivity: in brief, T1R are mainly linked to detection of sweet substances while T2R are mainly linked to the detection of bitter substances. Several research groups are currently attempting to characterize the type of receptor expressed by SCCs in the different organs; early results suggest that airway SCCs preferentially express T2R (bitter) receptors. In general, sweet taste receptors provide information about the caloric value of food, so they seem to be more directly related to food processing. In contrast, T2R receptors provide information about the presence of dangerous compounds that could represent a potential hazard for the mucosa.

Another marker that can be used for morphological identification of chemosensory cells is phospholipase C of the β2 subtype (PLC β2), which is expressed in a subset of cells within mammalian taste buds. This enzyme is believed to be a marker for gustatory sensory receptor cells (Kim *et al.*, 2006). IP3R3 and TRPM5 are other molecules that may be used to immunolocalize specific subsets of SCCs. Although these markers are common, the heterogeneity of the population composing the diffuse chemosensory system (DCS) makes unequivocal identification by a single marker difficult. To date, the utilization of protocols of chemical coding by co-localization of different elements of the chemoreceptorial molecular cascade seems to be the most promising technical approach.

Homology between the SCCs in different species

To establish homology among SCCs in fish, amphibians and mammals is difficult, partly because these cells form heterogeneous systems. So far, findings in mammals have generally confirmed previous findings in fish about the general morphology of SCCs, despite the fact that in mammals SCCs seem to be used as internal rather than as external chemoreceptors. In the oral cavity, homology between SCCs described in the different species seems evident, even if the relationship with the taste system requires further clarification. It is more difficult to determine homology in other parts of the body, in which complex end organs are lacking.

SCCs in the airway

In mammals, SCCs were first described in the oral cavity and seem to be widespread in large portions of the digestive apparatus. SCCs are also well represented in the respiratory apparatus, which shares a common endodermic origin with the digestive system. It has been shown that SCCs are diffusely present in the airways and in particular in the nasal cavity (Zancanaro *et al.*, 1999), where they detect irritants (Finger *et al.*, 2003). It was also demonstrated that these cells proliferate and undergo rapid turnover (Gulbransen and Finger, 2005). SCCs are also present both in the larynx (Sbarbati *et al.*, 2004 a,b) and in the trachea (Merigo *et al.*, 2005). These findings were obtained in rodents, which present very small airways in which the serous component largely prevails over the mucous component. Therefore, rodents are not ideal models for studying aspects that could be relevant for human pathology. Studies in species of large size and with respiratory mucosa resembling those of the human airway are in progress. One example to date is a study in *Bos taurus*, which demonstrated the presence of SCCs on the arytenoid epithelium, in the trachea and the bronchi (Tizzano *et al.*, 2006).

SCCs in the human nasal cavity

Data about SCCs in humans are scarce. Recent studies revealed a possible receptor cell in human and rodent olfactory epithelium. Also, electron microscopic studies of respiratory epithelium indicated several potential chemosensory cell types. Immunocytochemical experiments showed cell types positive for gustducin, calbindin and/or the vesicular acetylcholine transporter (VAchT) that closely resembled rodent SCCs (Hansen *et al.*, 2005). These cells have the morphology of SCCs and express Trp M5. Subsets of these cells express gustducin, calbindin and/or VAChT. These findings suggest the existence of possible unconventional receptor cell types in the respiratory epithelium of rodents and humans (Hansen *et al.*, 2006).

The specific laryngeal sensory epithelium

A specialized portion of the DCS seems to be located in the larynx. A specific laryngeal sensory epithelium (SLSE), which includes arrays of solitary chemoreceptor cells, has recently been described in the supraglottic region of the rat (Sbarbati *et al.*, 2004a). These SCCs lie in this specific epithelium together with taste buds. Recently, Finger *et al.*, (2005) demonstrated that taste buds are clearly innervated by nerve fibers immunoreactive for purinergic receptors, and that stimulation of taste buds *in vitro* evokes release of ATP. Thus, ATP fulfils the criteria for a neurotransmitter linking taste buds to the nervous system. On the other hand, laryngeal solitary chemoreceptor cells are not innervated by purinergic nerve fibers, although such fibers do innervate nearby epithelium. This indicates that nerve fibers that innervate laryngeal SCCs utilize a different neurotransmitter and/or receptor system (Finger *et al.*, 2005). The laryngeal immunoreactivity for α-gustducin was mainly localized in SCCs.

Laryngeal chemosensory clusters

In the larynx of the rat, a new form of chemosensory structure (i.e. the chemosensory cluster) has also been reported (Sbarbati *et al.*, 2004 b). These clusters are multicellular organizations which differ from taste buds and are generally composed of 2-3 chemoreceptor cells (Sbarbati *et al.*, 2004). Compared with lingual taste buds, chemosensory clusters show lower height and smaller diameter. In laryngeal chemosensory clusters, immunocytochemistry using antibodies against either α-gustducin or PLC β2 identified a similar cytotype. PLC β2 is expressed in a subset of cells within mammalian taste buds. The demonstration of the existence of chemosensory clusters strengthens the hypothesis of a phylogenetic link between gustatory and solitary chemosensory cells. Due to their structure and location, chemosensory clusters seem to represent the missing link between buds and SCCs. Laryngeal chemosensory clusters appear to be a transitional

structure between the rostrally located buds and SCCs, which are more distally located in specific areas of the larynx (Sbarbati *et al.*, 2004a).

In vivo approach by pharmacological magnetic resonance imaging

Considering the large amount of chemoreceptorial genes the capability of the chemosensory systems to recognize patterns of exogenous molecules is enormous. In the airway, the secretory responses to airborne molecules or to substances produced by microbial biofilms, which act on chemosensors may be evaluated by *in vivo* experimental paradigms using pharmacological magnetic resonance imaging. In such protocols, the integrity of the tissue is maintained such as the connectivity among several different cell types, the paracrine interaction, the blood flow and the innervation. Using this approach, we are testing on the airway, a large number of infochemicals extracted by bacteria, plants or animals. The preliminary results confirm the possibility that the airway secretion may be controlled by chemical cues.

The DCS and bacterial chemosensory systems

The presence of a DCS in the airways raises questions about the role of chemoreceptors in control of complex functions (e.g. airway surface liquid secretion) and about the involvement of chemoreceptors in respiratory diseases.

The chemoreceptive capacity of the DCS seems to protect against exogenous substances. In addiction, recently published data suggest that the DCS could have an important role in defense against bacteria. The elements of this system are located in an optimal position to intercept the exchange of information between bacteria operated by the quorum sensing strategy (Kolter, 2005). Briefly, these bacteria co-ordinate their activities using extracellular signals, i.e. auto-inducers or pheromones (Hardman *et al.*, 1998). When such compounds reach a sufficient concentration (i.e. when the total population is large enough), the bacteria activate genetic pathways often involved in the initiation of aggressive behavior. Quorum sensing appears therefore to be a strategy used by bacteria to co-ordinate their activities, and it is based on the release of small molecules, generally proteins or acyl-lactones. These findings suggest that a *war of communication* takes place on the mucosal surfaces of the digestive and respiratory systems, with two chemosensory systems in opposite camps (Sbarbati 2006; Sbarbati and Osculati, 2006).

Due to its structural and biochemical characteristics, the DCS appears to be able to intercept communication among bacteria and predict their movements. If messages are indeed detected in this way, it may be that the organism mounts a highly localized and efficient response to bacterial activation. This would be based on defenses like the quenching of auto-inducers, the dilution or removal of bacteria, or secretion of antibiotic agents, and it might precede or avoid the need for intervention by immune cells.

The inflammatory reflex

It is well known that in the airway, the control of inflammation is based on information provided by vagal sensory afferents and that central integration devices operate between sensory and effector structures, which could act on both immune and mucosal cells (Figure 2) (Andersson, 2005). This inflammatory reflex is a physiological pathway in which the nervous system detects inflammatory stimuli and modulates cytokine production. Afferent signals to the brain are transmitted by the vagus nerve, which activates a reflex response that culminates in efferent vagus nerve signalling. Termed the *cholinergic anti-inflammatory pathway*, efferent activity in the vagus nerve releases acetylcholine that interacts with macrophage nicotinic receptors (Czura and Tracey, 2005).

In the past, the afferent input was considered to be generated by vagal free nerve endings but the new data demonstrated that the SCCs forming the DCS may be innervated. Therefore, further studies must evaluate whether these specialized epithelial elements significantly contribute to the vagal input in the context of the inflammatory reflex.

The intramucosal reflex

In addition to central reflexes, further defensive lines against micro-organisms and xenobiotics are based on intramucosal reflexes. Figure 1 schematically illustrates the cell types putatively involved. Our preliminary results suggest that activation of the DCS leads to a secretory response by the mucosa and activation of mucociliary clearance (Merigo *et al.*, 2007). In particular, bitter substances can stimulate a secretory reflex that is in part supported by a chemoreceptorial capacity of secretory cells (short reflex). The increased activity of mucosal cells may result in dilution of bacterial

quorum sense signals and their removal by mucociliary clearance.

The relationship between SCCs and brush cells

Brush cells (BCs) are elements characterized by a brush of rigid apical microvilli with long rootlets, which are found in the digestive and respiratory apparatuses. In the past, these cells have been given names such as tuft, fibrillovesicular, multivesicular or caveolated cells.

The first description of BCs is generally attributed to Rhodin and Dalham (1956) in the rat trachea. Since the first description, the presence of BCs has been confirmed in the airway of several species, including humans (Rhodin, 1959). BCs were then detected in the lung (Meyrick and Reid, 1968) and in the digestive apparatus (Luciano *et al.*, 1968 a,b), mainly in the gallbladder (Luciano and Reale, 1969). The recent description of gustducin (Hofer and Drenckhahn, 1998) and other bitter-taste related molecules in BCs in the digestive and respiratory apparatuses demonstrated a link between these cells and elements of taste buds. The recent results support the idea that BCs may operate as solitary chemoreceptors (Sbarbati and Osculati, 2005), probably representing a subfamily of SCCs localized in specific microenvironments.

Conclusions

Several questions remain about SCCs and about the physiology and morphology of the DCS. In particular, the links between the molecular mechanisms of taste and secretory apparatuses have not yet been studied, and the existence of BCs not containing α-gustducin raises the possibility of alternative G-proteins. Such questions could be answered by a detailed chemical code for the different elements of the DCS.

This DCS seems to be a potential new drug target because several elements indicate that information obtained by this system induces secretory reflexes. Therefore, modulation of the respiratory and digestive apparatuses by substances acting on their chemoreceptors could be important in the treatment of diseases such as cystic fibrosis and asthma, and might open new frontiers in drug discovery.

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The modality of transendothelial passage of lymphocytes and tumor cells in the absorbing lymphatic vessel

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The modality of transendothelial passage of the macromolecules and cells (lymphocyte and cancer cells) in the absorbing lymphatic vessel (ALV) and the tumor-associated absorbing lymphatic (TAAL) vessel is studied. On the basis of the peculiar plasticity of the lymphatic endothelial cell of these vessels (lacking a continuous basement membrane, pores and open junctions) the endothelial wall organizes formation of the *intraendothelial channel*, by means of molecular interactions as yet unidentified. The remarkable finding of the intravasation of lymphocyte and experimental tumor cancer cells (T84 colon Adenocarcinoma, B16 melanoma in nude mice and spontaneous prostate adenocarcinoma in transgenic mice) should be stressed. This intravasation takes place, under both physiologic and pathological conditions, following the same transendothelial morphological modality, i.e. the intraendothelial channel – a dynamic and transient entity - is probably also induced by similar molecular interactions, a crucial point that merits future research.

Key words: lymphatic, intravasation, lymphocyte, cancer cell, metastasis, transendothelial migration.

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The morphological findings obtained in the second half of the 1900s regarding what composes the canalization of the lymphatic vascular system (LVS) helps clarify not only the LVS's role, complementary to the blood vascular system, but also its importance in lymphocyte homing, in regulating tissue homeostasis and in some interstitial matrix pathologies. Furthermore, the fine structure of the vessels of the LVS allows us to distinguish two distinct sectors composed of (a) lymphatic vessels whose main function is that of lymph conduction and flow (pre- and post-lymph nodal collector, lymphatic trunk vessels, thoracic duct); (b) lymphatic vessels with high absorption capacity (the chyliferous vessel; vessels of the mucosal, submucosal and muscular network) (Ottaviani and Azzali, 1965). The latter, unlike collector vessels characterized by a monolayer of endothelial cells that rests on a continuous basement membrane covered externally by one or more strata of smooth muscle fibers, are lacking a continuous basement membrane, pores and open junctions. Furthermore, the abluminal surface of endothelial cells establishes an extensive connection with the components of the extravasal interstitial matrix. The recent use of specific markers to detect the lymphatic endothelium [LYVE-1, Prox-1, tetraspanin, podoplanin, D2-40 (Jackson *et al.,* 2001; Longo *et al.,* 2001; Prevo *et al.,* 2001; Kahn *et al.,* 2002)] and the lymphangiogenesis induced by growth factors [VEGFR-3, VEGF-C and VEGF-D, etc. (Achen *et al.,* 1998; Swartz and Skobe, 2001; Sleeman, 2001; Stacker *et al.,* 2002)] revived interest in the biologic potentiality of the lymphatic vessel. Despite the prestigious results obtained, information regarding the mechanisms that regulate the transendothelial passage of macromolecules and cells into the absorbing lymphatic vessel (ALV), is still lacking. In recent decades, the prevailing view on this subject sustains the hypothesis of the open junction resulting from stretching of the anchoring fibers (Casley-Smith,

1964; Leak and Burke, 1968; Castenoltz, 1984), and of the vesicular pathway for particles suspended in the interstitial fluid (O'Morchoe *et al.,* 1985). Azzali demonstrated (1982-1999), under physiologic and seasonal conditions of various animals, that the macromolecule intravasation occurs through the so-called intraendothelial channel that the absorbing lymphatic endothelium itself organizes due to stimuli and interactions not yet defined. The morphological aspect of the intraendothelial channel resembles that of a mountain tunnel 7.2 m long and 1,8-2 m in diameter, with an abluminal and a luminal orifice. Following the variations in its numerical density under normal and experimental conditions (fasting, seasonal cycle in hibernating animals, lymph stasis after binding of the prelymph nodal collector vessels, etc.) this channel should be considered a dynamic morphological entity that plays a pivotal role in lymph formation as well (Azzali, 2003).

Concerning cell intravasation (lymphocyte, leukocyte) into the absorbing lymphatic vessel, the modality of cell entering, the interactions and transport into the vessel must still be clarified, while the hypotheses formulated on high endothelial venules (HEV) of the blood vascular system are numerous and detailed. For the transendothelial passage of lymphocyte and leukocyte in the lymphatic vessel Carr *et al.,* (1975) propose the interendothelial junctions pathway, but unfortunately the mechanisms that regulate their opening and closing are still unknown (Dejana *et al.,* 2006); Ohtani *et al.,* (1986) and Kato (1988) sustain the hypothesis of a transendothelial migration without however making any reference to the migratory mechanism. Nieminen *et al.,* 2006 suggest that the para- or transcellular migratory pathway could be cell-specific, where the transcellular way would be exclusively for the lymphocyte, while the neutrophil would use the intercellular way. According to Mamdouh *et al.,* 2003, the transcellular migration of the leukocyte could occur following its being enveloped by an endothelial cell, or by englobing microvilli rich in vimentin (transmigratory cup, proposed by Carman *et al.,* 2004). Through observation of ultrathin serial sections of lymphatic vessels having englobed cells in their endothelium, and their three-dimensional reconstruction, we demonstrated that the transendothelial migration of the lymphocyte and leukocyte, even in a modest inflammatory state, occurs only at the level of the lymphatic vessel with high absorbing capacity, and not in lymphatic vessels whose prevailing function is that of lymph conduction and flow. The lymphocyte would migrate from the extravasal matrix toward the lymphatic vessel under the influence of the microenvironment (Entschladen *et al.,* 2004), growth factors, and degrading enzymes (proteases, metalloproteases). The direction of the migratory process is coordinated by cytoplasmatic protrusions, especially ondulopodium-like, whose formation is guided by the polymerization of ectoplasmatic actin filaments. This pseudopodium would be encircled by a ring of ICAM-1, F-actin and caveolin (Millan *et al.,* 2006) sets that would also act to recognize the area of the endothelial wall prepared for adhesion and intravasation (Figure 1). The lymphocyte, after having established close adhesion with the endothelial wall due to the bonding between L-selectin and the Mannose receptor (Irjala *et al.,* 2001), enters the vessel lumen through the intraendothelial channel in the chyliferous vessel and in the lymphatic vessels of the small intestine submucosal network (Figures 2 and 3). This channel would be modulated by the endothelial wall on biomolecular bases not yet defined, without involving interendothelial contact. A similar modality of transendothelial migration was confirmed also in our recent studies on the ALV in interfollicular areas of Peyer's patches lymphoid tissue, in the vermiform appendix of different micromammals and in the lymphatic vessels of the choriallantoic membrane of 18-day-old chick embryos.

Concerning the tumor cell intravasation in the tumor-associated lymphatic (TAAL) vessel in the tumor mass derived from melanoma B16 and colon adenocarcinoma T84 cell xenografts in nude mice, we demonstrated that the lymphatic endothelium has the same ultrastructural characteristics as the ALV described in normal tissues and organs (Azzali, 2006). The tumor cell population is formed of stromal tumor cells (CT) and invasive phenotype tumor (IPT) cells distributed in a disorganized manner in the extravasal matrix, and only IPT cells, by an active collective or individual movement toward the lymphatic vessel, can reach the endothelial wall (Figure 4). In this migratory movement there is a multistep cascade of interactions between surface molecules of the IPT cell and their counterreceptor in the lymphatic endothelium. This migratory movement through the extravasal matrix (ECM) provoked by invadopodia, composed of

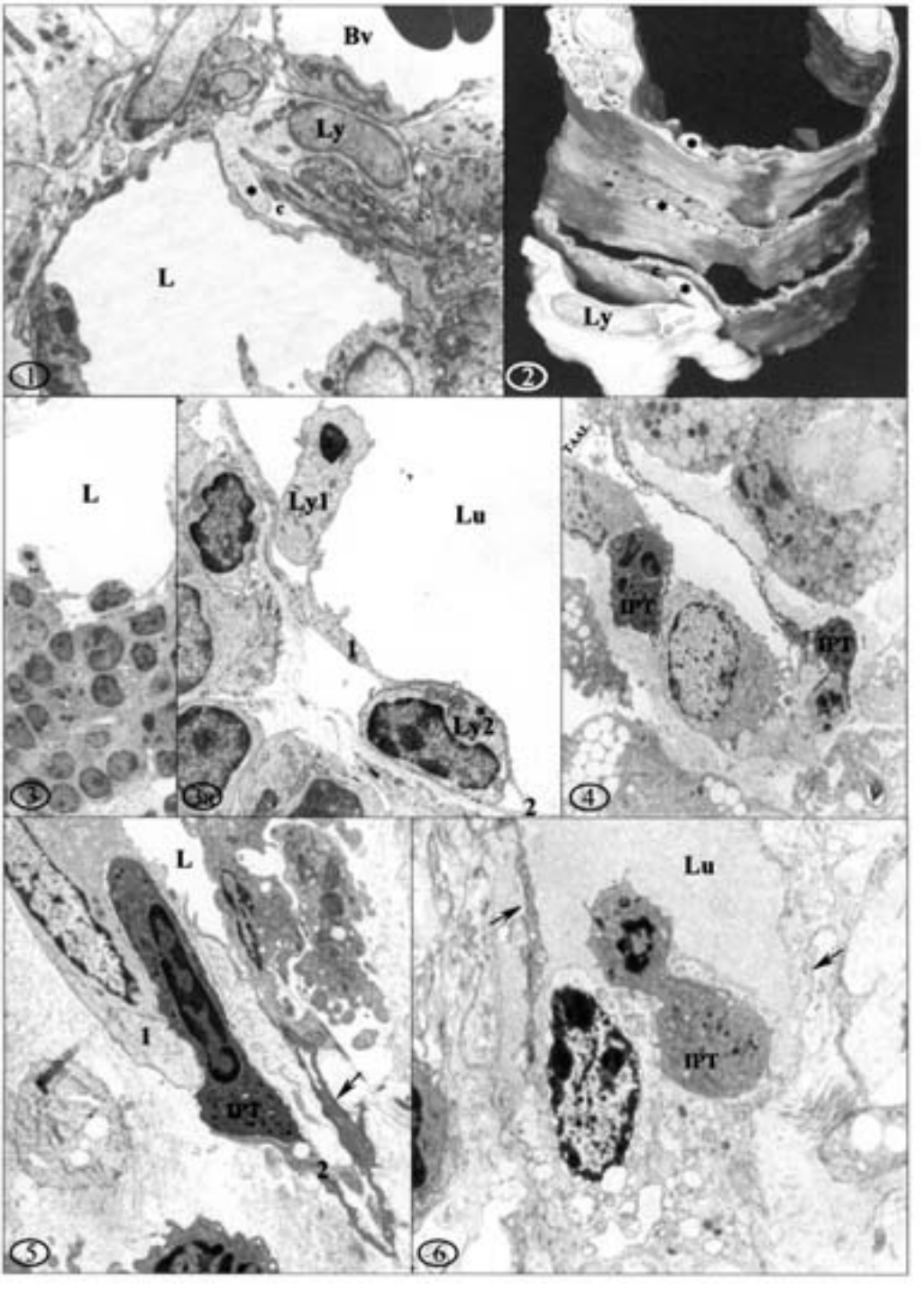

Figure 1. Absorbing lymphatic vessel (L) with cytoplasmatic expansion (*) of a lymphocyte (Ly) wedged in the abluminal orifice of an intraendothelial channel (c). Bv: blood vessel with erythrocytes. × 38000, 1/3 original magnification.

Figure 2. Three-dimensional model derived from ultrathin serial sections of the absorbing lymphatic vessel of Figure 1, to demonstrate in cross-section the route of the cytoplasmatic expansion (*) of the lymphocyte (Ly) inside the intraendothelial channel.

Figure 3 and 3a. Lymphatic vessel (L) of the interfollicular area of a Peyer's patch with a lymphocyte migrated into the vessel lumen (Lu) through the luminal orifice of the intraendothelial channel. In Ly2 a lymphocyte englobed between the cytoplasmatic expansion of endothelial cell 1 and the secondary extension (2) of the endothelial cell 2. × 11000, 1/3 original magnification.

Figure 4. Tumor cells evolved in the invasive phenotype (IPT) distributed in proximity to the endothelial wall of a tumor-associated absorbing lymphatic vessel (TAAL). × 8000, 1/3 original magnification.

Figure 5. TAAL vessel (L) with IPT cell wedged in an intraendothelial channel formed by endothelial cell 1 cytoplasm and by the secondary extension (2) of the adjacent endothelial cell (arrow), Lu = TAAL vessel lumen. × 10000, ½ original magnification.

Figure 6. TAAL vessel with IPT cell inside an intraendothelial channel under the sagittal section plane, whose apical cytoplasm is already in the lymphatic vessel lumen (Lu). Arrows = TAAL vessel endothelial wall. x 11000, 1/2 original magnification.

membrane proteins such as actin, N-WASP, cortactin and ECM degradation enzymes would be favored, according to Yamaguchi and Condeelis (2006), by chemoattractants secreted by vasoactive cells (Condeelis and Segall, 2003) or by SLC CCL21, which guides the directional migration (Muller, 2002; Nathanson, 2003) released by the lymphatic endothelium (Gunn *et al.,* 1999). When the IPT cell reaches the endothelial wall of the TAAL vessel it adheres to it firmly (Figure 5), following interactions which modulate the CT-endothelial lymphatic cell adhesion of L-selectin, 18 integrin and 18 integrin, and of the Ig superfamily such as MCAM, JAM2 (Wolf *et al.,* 2003). This adhesion takes place after recognition of the lymphatic endothelium area prepared by bidirectional interactions between the IPT cell and adjacent stromal cells (fibroblasts, endothelial cells, immune cells) that are still unknown. Concerning the way of the metastatic dissemination of IPT cell from the primary site, it is generally thought that it occurs a) by the peritumoral lymphatic vessels invasion due to high pressure into the tumor mass (Carmeliet and Jain, 2000; Williams *et al.,* 2003); b) by the formation of new lymphatic vessels (lymphangiogenesis) induced by the VEGF-C and VEGF-D overexpression (Stacker *et al.,* 2002). Serial sequence of the ultrastructural pictures showing different moments of the migration process of the IPT cell through the endothelial wall and their reconstruction in three-dimensional wax models made it possible to demonstrate formation of the intraendothelial channel, through which the IPT cancer cell's intravasation into lymphatic circulation occurs (Figure 6). This channel presents the same morphological features documented in the absorbing lymphatic vessel of man, several micromammals and birds (Azzali, 2003). As a result of these findings, a reliable answer to the questions postulated by Stacker *et al.,* 2002; Skobe *et al.,* 2001; Padera *et al.,* 2004 etc., on the modality of transendothelial migration of the cancer cell is provided for the first time. Furthermore, the IPT cell route inside the TAAL vessel and from there into the prelymph nodal collector vessel up to parenchima level of the satellite lymph node, underlines the active role played by the lymphatic pathway in metastatic diffusion. Recently, these morphological findings obtained for melanoma B16 and T84 colon Adenocarcinoma xenografts, were also confirmed in prostatic Adenocarcinoma and the seminal vesicle metastasis tumor mass in transgenic mice (*data not yet published*).

These original findings regarding the modality of the transendothelial passage (intravasation) of the cell lead us to make some interesting observations:

a. We have demonstrated *how* cells establish adhesion to the lymphatic endothelium, which in its turn organizes, independently of end to end, overlapping and interdigitating interendothelial contacts, the intraendothelial channel. This is a morphological, dynamic and transient entity which changes its numerical density under certain experimental and physiological conditions and plays a crucial role in immune response (lymphocyte homing) and in cancer cell metastatic dissemination.

b. The intraendothelial channel is a concrete answer to hypotheses formulated regarding intravasation modality in the lymphatic circulation of the lymphocyte and cancer cell. Moreover, this intravasation differs from the multiple factors pathway (Cao *et al.,* 2004), from the intraendothelial way via open junctions with anchoring filaments of fibrillin (Gerli *et al.,* 2000) and from the non-destructive way of the endothelial cell, proposed by Timar *et al.,* 2001.

c. The transendothelial migration of macromolecules and cells occurs only in the endothelium of the peritumoral lymphatic vessel with absorbing capacity, since intratumoral vessel would not be functional. Furthermore, the morphological mechanism of intravasation is the same for both lymphocytes and cancer cells; this is an interesting functional peculiarity of the lymphatic endothelium as compared to postcapillary venules of blood circulation.

d. The lack of knowledge concerning the molecular bases that induce the organization of the intraendothelial channel by the absorbing lymphatic endothelium is critically important and a stimulus for future research. Once acquired, this knowledge would open new therapeutic strategies for fostering or blocking the formation of the intraendothelial channel, for the benefit of certain pathologies of the extracellular matrix (lymphedema) and for preventing metastatic dissemination of the cancer cell.

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Scatter factor-dependent branching morphogenesis: structural and histological features

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Branching morphogenesis is a multi-step process that controls the formation of polarised tubules starting from hollow cysts. Its execution entails a series of rate-limiting events which include reversible disruption of cell polarity, dismantling of intercellular contacts, acquisition of a motile phenotype, stimulation of cell proliferation, and final re-establishment of cell polarity for creation of the definitive structures. Branching morphogenesis takes place physiologically during development, accounting for the establishment of organs endowed with a ramified architecture such as glands, the respiratory tract and the vascular tree. In cancer, aberrant implementation of branching morphogenesis leads to deregulated proliferation, protection from apoptosis and enhanced migratory/invasive properties, which together exacerbate the aggressive features of neoplastic cells. Under both physiological and pathological conditions, branching morphogenesis is mainly accomplished by a family of growth factors known as scatter factors. In this review, we will summarise the current knowledge on the biological and functional roles of scatter factors during branching morphogenesis, with a special emphasis on the phenotypic (structural and histological) consequences of scatter factor activity in different tissues.

Key words: branching morphogenesis; scatter factors; cell adhesion and motility; tyrosine kinases.

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Scatter factors, scatter factor receptors, and branching morphogenesis

Branching morphogenesis is the morphological counterpart for a functional process known as *invasive growth* and identifies a physiological genetic programme which is controlled by a family of soluble signals known as Scatter Factors. Under normal conditions, this programme leads to morphogenetic movements and a change in the three-dimensional organisation of tissues at the time of development and organ regeneration. When the invasive growth programme is executed in an abnormal manner, rampageous cell proliferation, uncontrolled migration, and resistance to programmed cell death occur in the tissues and organs. Together, such aberrant processes recapitulate most of the characteristics of cancer malignancy.

Scatter Factor (SF) has the ability to induce intercellular dissociation of epithelial cultures within a few hours after administration (hence its name), and was found in the 1980's to be secreted by fibroblasts in culture (Stoker *et al.,* 1987) (Figure 1). The same protein, isolated from platelets or from the blood of patients with acute liver failure (Nakamura *et al.,* 1986, Zarnegar and Michalopoulos, 1989), has been shown to be a potent growth factor for hepatocytes in culture. Due to this activity, SF is also named hepatocyte growth factor (HGF) (Nakamura *et al.,* 1989). Thus, in the following treatise, we will indicate SF with the acronym SF/HGF. The SF family also includes the macrophage stimulating protein (MSP or SF-2). SF/HGF is the ligand for the tyrosine kinase receptor encoded by the proto-oncogene MET (Bottaro *et al.,* 1991, Naldini *et al.,* 1991), while MSP binds a receptor highly homologous to MET, encoded by the RON oncogene (Gaudino *et al.,* 1994). Interestingly, a further member of the MET receptor family (SEA) was demonstrated to be the avian counterpart of RON (Huff *et al.,* 1993, Wahl *et al.,* 1999). RON also mediates epithelial cell scatter

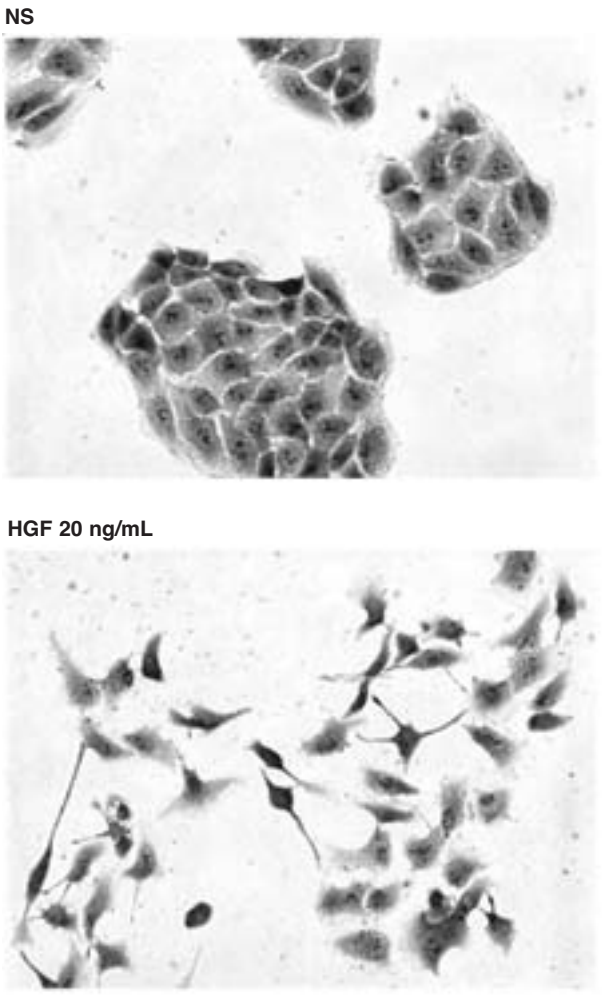

Figure 1. *The Scatter effect*. Epithelial cells (MDCK, canine renal cells) grow as compact islands (NS, non stimulated). Addition of SF/HGF to the culture medium induces cell dissociation and acquisition of a mesenchymal phenotype (micrographs, 200x).

and proliferation, in a fashion that is similar to MET (Medico *et al.,* 1996).

Again in the 1980's, through the study of human cell lines treated with chemical carcinogens, MET was identified as a transforming oncogene, activated by translocation and fusion with the TPR gene (Cooper *et al.,* 1984) (Park *et al.,* 1986). TPR-MET includes most of the MET intracellular tyrosine kinase domain, which is constitutively dimerized, and thus activated, through a leucine-zipper domain provided by TPR (Rodrigues and Park, 1993).

Analysis of MET expression and activity in patients and in experimental systems, highlighted the unconventional nature of this oncogene. Indeed, MET activation causes not only transformation but also an invasive and motile phenotype *in vitro* and metastatic spread after *in vivo* cell transplantation (reviewed in: Birchmeier *et al.,* 2003, Trusolino and Comoglio, 2002).

Structural and cellular aspects of branching morphogenesis

The concept of *Invasive Growth* as the functional expression of branching morphogenesis has arisen from the clarification of the role of MET signalling in embryonic development and cancer progression (Comoglio and Trusolino, 2002). Accordingly, several studies have highlighted a particular pattern of behaviour stimulated by SF in a number of different cell types and in a range of different biological contexts.

SF-induced invasive growth is highly regulated and is commonly seen during the formation of ramified tubules and papillary outgrowths that make up the parenchymal architecture of epithelial organs (for example, liver and kidney) (Brinkmann *et al.,* 1995, Woolf *et al.,* 1995), or during the development of the blood circulatory tree (vasculogenesis and angiogenesis) (Bussolino *et al.,* 1992). Interestingly, specialised facets of invasive growth can be observed in the nervous system, where, upon Scatter Factor stimulation, axons extend through tissues to reach their final synaptic target (the so-called *axon guidance*) (Ebens *et al.,* 1996); in the bone marrow, where haemopoietic precursors dissociate from their niches and are released into the blood circulation (Galimi *et al.,* 1994); and finally in bone, when osteoclasts proliferate and penetrate the mineralized matrix in order to modify the tissue (Grano *et al.,* 1996).

Branching morphogenesis stimulated by SF is conducted through a series of stages that have been characterized in detail through *in vitro* studies (Montesano *et al.,* 1991, Medico *et al.,* 1996) (Figure 2). This process is initiated by the formation of cysts from epithelial cells resuspended in a three-dimensional matrix. These cysts emerge as spherical monolayers of polarized cells that encapsulate a central lumen.

Cells extend long protrusions into the surrounding matrix, followed by the movement of some cells along the pathway opened by the protrusion. This results in a loss of polarity whilst retaining only minimal intercellular contacts. The ensuing disposal of cells into multi-layered cords, re-formation of junctions and polarization and, ultimately, the

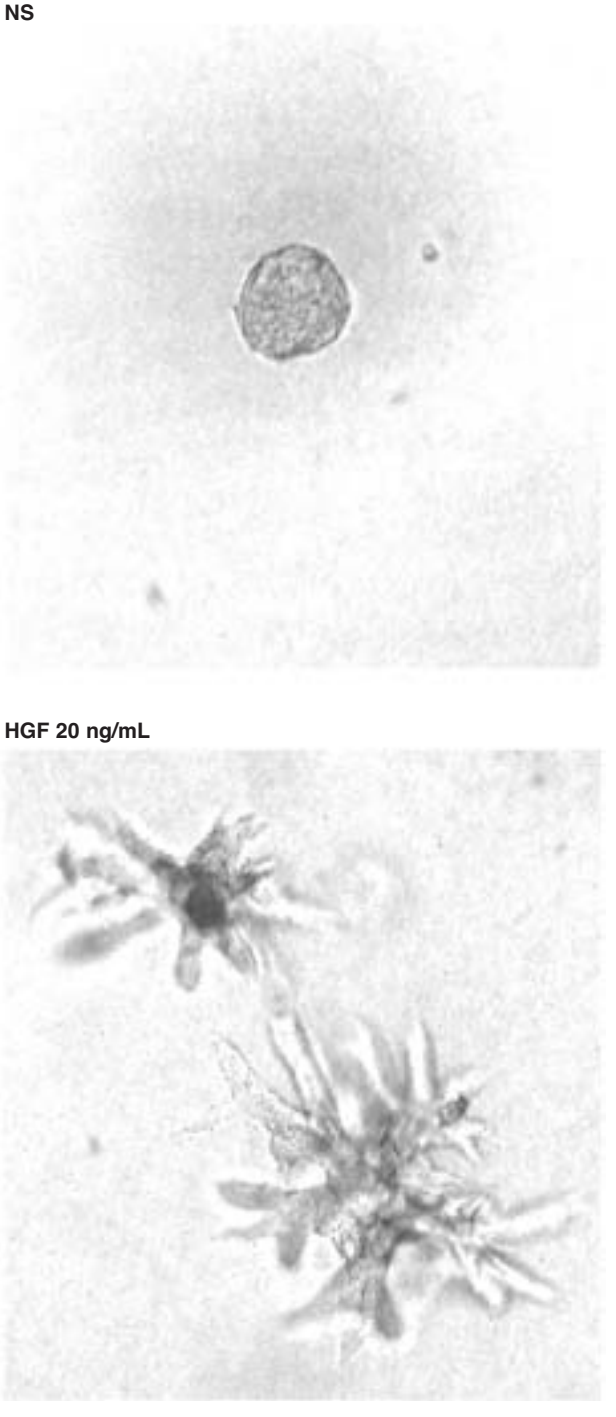

Figure 2. ***Branching morphogenesis.*** **Epithelial cells (MLP29, mouse liver progenitors) resuspended in a tridemensional collagen matrix form spheroids (NS, non stimulated). Addition of SF/HGF to the culture medium induces the cells to emit protrusions, migrate along them, and rearrange into hollow branching tubular structures lined by polarized cells (micrographs, 10x).**

establishment of a new central lumen all result in branched tubular structures, which represent the morphological endpoint of epithelial tubulogenesis (reviewed in (O'Brien *et al.,* 2002).

When cells are grown on a bi-dimensional plastic support, upon SF stimulation, the cells break down their junctions and move off in all directions. Similarly, cells seeded on an artificial basement will migrate across it (Weidner *et al.,* 1990). This invasive/motile response becomes constitutive when cells express an activated MET oncogene or display chronic SF/HGF signalling (reviewed in: Trusolino and Comoglio, 2002). Moreover, protection from apoptosis occurs in to cells that have been stimulated by SF/HGF (Bardelli *et al.,* 1996). This is extremely important during cancer progression and metastatisation, as tumour cells emigrated from the primary tumour mass and navigating in foreign tissues must resist the pro-apoptotic stimuli that previously unexplored environments exert on them (Mehlen and Puisieux, 2006).

Branching morphogenesis in the embryo

The ability to perform invasive growth seems inherent in undifferentiated, stem and progenitor cells of the embryo. During development, morphogenetic movements depend on the conversion of epithelial cells to a mesenchymal and motile phenotype which is suitable for migration through the extracellular environment and organisation in multi-layered organs which eventually incorporate several tissues. This process, known as the *epithelial-mesenchymal transition*, takes place immediately after the formation of the primitive streak and in co-incidence with the initial cell activities aimed at transformation of the flat organism into a three-dimensional one (reviewed in: Thiery, 2002). This transformation implies a *Scatter effect* in which, conceivably, SF play an important role.

In early embryos SF/HGF is expressed by the Hensen's node and in endodermal and mesodermal structures along the rostro-caudal axis (Andermarcher *et al.,* 1996, Streit *et al.,* 1995). In these primitive tissues SF/HGF likely acts in an autocrine/paracrine fashion. During the ensuing organogenesis, paracrine stimulation becomes the rule, as SF/HGF and its receptor are expressed in a dynamic and complementary pattern: in general, epithelial cells express the receptor, while cells of mesodermal origin secrete the factor (reviewed in: Birchmeier and Gherardi, 1998).

In the mouse, the SF/HGF signalling system is present and active throughout many embryonic tissues and organs, and during the entire developmental process (Sonnenberg *et al.,* 1993). Genetic

analysis of mice has shown that SF/HGF and its receptor are an absolute requirement for the development of specific organs. In knock-out mice ablation of SF/HGF or MET is lethal to the embryo resulting in impaired formation of the labyrinthine layer of placenta, the liver, and the diaphragm and limb muscles (Bladt *et al.,* 1995, Maina *et al.,* 1996, Schmidt *et al.,* 1995, Uehara *et al.,* 1995). Interestingly, SF/HGF is expressed throughout the myoblast pathway and controls cell delamination from somite-derived axial structures (dermomytomes) and cell directional migration towards peripheral limb buds (reviewed in: Birchmeier and Gherardi, 1998, Birchmeier *et al.,* 2003)

The structure of scatter factors and their receptors

Mature, biologically active SF have an atypically large size (94kDa). They consist of two disulphide-linked chains (α and β). The α chain is characterized by the presence of an N-terminal *hairpin loop*, followed by four *Kringle Domains* (80-amino acid double-looped structures stabilized by internal disulphide bridges). These *Kringle Domains* are a common feature of plasminogen-related proteins (Nakamura *et al.,* 1989). The β chain is homologous to serine-proteases (like plasminogen and clotting factors) but lacks proteolytic activity, owing to substitution of three aminoacidic residues critical for catalytic functions (Nakamura *et al.,* 1989). Thence, SF have an interesting relationship with constituents of the blood clotting cascade as they are philogenetically related to plasminogen, a circulating proenzyme whose active form, known as plasmin, is responsible for fibrinolysis (degradation of blood clots).

SF are similar to coagulation proteins both in their structure and in their mechanism of activation. Both SF/HGF and MSP are secreted as single-chain inactive precursors (pro-HGF and pro-MSP) and are activated by a proteolytic cleavage which is performed by proteins also involved in clotting regulation. The first enzyme to be shown as a potent activator of pro-HGF was urokinase-type plasminogen activator (uPA) (Naldini *et al.,* 1992, Mars *et al.,* 1993). This was followed by evidence that coagulation factor XII, thrombin and one serine-protease (XII- like factor) also function as HGF convertases (Shimomura *et al.,* 1993, Shimomura *et al.,* 1995).

SF/HGF binds heparin-sulphate proteoglycans, which provide an extracellular reserve of the factor *in vivo* and limit its diffusion through extracellular fluids. This, in turn, promotes SF/HGF sequestration on proximity to the site of synthesis and a paracrine-like mode of activity (Hartmann *et al.,* 1998). Proteoglycans are not necessary for SF/HGF binding to its receptor, however they couple SF/HGF in symmetrical dimers that simultaneously engage two receptor molecules, thus inducing receptor dimerisation and trans-activation (Chirgadze *et al.,* 1999, Schwall *et al.,* 1996).

The SF/HGF receptor, encoded by MET, and the MSP receptor, encoded by RON, sharing approximately 60% homology, are disulphide-linked α/β heterodimers that form by intracellular proteolytic processing of a single-chain precursor. In both receptors the α subunit is completely extracellular, while the β subunit is a single-pass transmembrane chain encompassing the tyrosine kinase activity. A peculiar structural motif, the *Sema Domain*, characterizes the extracellular region of SF receptors. The Sema Domain contains over 500 amino acids, inclusive of the full α chain (approximately 300 amino acids) and the amino-terminal moiety of the β chain. In recent mutagenesis studies of MET, it has been demostrated that the Sema Domain is equipped with low-affinity ligand binding (Gherardi *et al.,* 2003).

In the extracellular portion there is also a cystein rich region and a string of four immunoglobin-like structures that are typical protein-protein interaction domains.

The intracellular domain of the SF receptors is composed of a tyrosine-kinase catalytic site surrounded by juxtamembrane and carboxy-terminal regulatory regions. Residues involved in receptor downregulation are found in the juxtamembrane domain. These include: (a) a serine residue (S985), whose phosporylation inhibits tyrosine kinase activity; (b) a tyrosine residue (Y1003), which, upon phosporylation, associates with the protein adaptor CBL, which is an essential intermediary of MET quantitative downregulation. Two separate receptor-degradation pathways are switched on by CBL. One of these is controlled by ubiquitination (CBL is a E3 ubiquitin-ligase) whilst the other is controlled by endocytosis (CBL recruits regulatory components of endocytic vesicles) (reviewed in: Trusolino and Comoglio, 2002).

In the MET carboxy-terminal domain there is a conserved sequence (Y^{1349}VHV---Y^{1356}VNV) that

includes two critical tyrosines for MET signalling. Following phosphorylation, such tyrosines generate a comprehensive docking site for signal transducers. In general, tyrosine kinase receptors use different phosphotyrosines to engage distinct SH2-containing signal effectors; in the case of MET, the above mentioned sequence is capable of engaging the complete spectrum of transducers that are necessary for invasive growth, hence it is called *multi-functional docking site* (Ponzetto *et al.,* 1994). Indeed, when the two tyrosines of the multi-functional docking sites are replaced by phenylalanine cells cease to respond to SF/HGF (Ponzetto *et al.,* 1994).

Genetic *in vivo* experiments have demonstrated the importance of these residues for receptor biological functions. A transgenic mouse expressing a receptor in which the two tyrosines of the multi-functional docking site have been phenylalanine-permutated exhibits a lethal phenotype akin to that of the MET null-mouse (Maina *et al.,* 1996). Similarly, mutation of the same tyrosines annuls the activated MET signalling and oncogenic properties without affecting the tyrosine kinase function (Bardelli *et al.,* 1998). This indicates that the tyrosine kinase activity of MET is meaningful for signalling only when the receptor can properly associate to signal transducers. Therefore, the multi-functional docking site is the force that drives the biological properties of the SF/HGF receptor, and is likely to be responsible for its specific ability to induce branching morphogenesis. Accordingly, when inserted into the intracellular domain of other receptor tyrosine kinases, the multi-functional docking site bestows the ability to transduce the branching morphogenic signals even to receptors that usually induce only cell proliferation (Sachs *et al.,* 1996).

Signal transduction: from private adaptors to multiple co-receptors

Several SH2-containing signal transducers can be recruited by the multi-functional docking site at high affinity. This ability, which is remarkable and unique among tyrosine kinase receptors, is based on the wide range specificity of the consensus sequence formed by the two phosphorylated tyrosines (Y^{1349} and Y^{1356}) and the three aminoacids that follow each tyrosine (Ponzetto *et al.,* 1994). Thus, MET can concurrently activate multiple signal transduction pathways, including GRB2-RAS, phosphatidylinositol-3 kinase (PI3-K), SRC and signal transducer and activator of transcription (STAT) (Boccaccio *et al.,* 1998, Graziani *et al.,* 1991, Ponzetto *et al.,* 1994).

Activation of RAS plays a key role in the biological activities induced by MET, affecting both cell scattering and proliferation. When the RAS transduction pathway is impaired by expression of a dominant-negative RAS (Hartmann *et al.,* 1994) or by micro-injection of neutralizing antibodies, an inhibition in the motile response to SF/HGF occurs. Furthermore, direct activation of the RAS pathway by MET, through association of the GRB2 adaptor to the multi-functional docking site, is an important determinant of cell proliferation and therefore also of the MET oncogenic potential. In fact, mutagenesis of the MET multi-functional docking site that selectively abrogates GRB2 recruitment, interrupts the proliferative signals and abolishes the transforming potential of the activated MET oncogene (Ponzetto *et al.,* 1996). However, the same MET mutant retains an unaffected ability to induce cell motility, showing that the threshold signal to achieve this part of the invasive growth response is nevertheless achieved (Ponzetto *et al.,* 1996). As a whole, activation of the RAS pathway by MET is an absolute requirement for branching morphogenesis (Fournier *et al.,* 1996). Sustained activation of ERK 1/2 MAP kinases, which are downstream RAS effectors, has recently been highlighted as a specific feature of morphogenesis (Boccaccio *et al.,* 2002, O'Brien *et al.,* 2004). The ability to sustain prolonged MAP kinase activation sets SF/HGF apart from pure mitogens that evoke only a transient peak of MAP kinase activity (Marshall, 1995).

Another crucial signal transducer of MET is PI3-K, which is activated by direct recruitment through the multi-functional docking site or as a RAS effector (Graziani *et al.,* 1991, Ponzetto *et al.,* 1993). PI3-K is essential for cell scatter *in vitro*, being necessary for disassembly of intercellular contacts {Potempa and Ridley, 1998, Royal, and Park, 1995} and remodelling of adhesion to the extracellular substrate (Trusolino *et al.,* 2000). Signalling pathways downstream from this transducer are engaged in either cytoskeletal reorganization and motility (such as the small GTPase Rac and the protein kinase PAK) (Royal *et al.,* 1997), or in protection from apoptosis (such as AKT) (Xiao *et al.,* 2001). A form of apoptosis, known as anoikis, can occur

when a cell loses its normal adhesive contacts. Therefore, protection from this form of apoptosis is imperative when a cell detaches from its primary site and travels through the extracellular matrix (ECM). It is not surprising, then, that activation of PI3-kinase by MET is required to accomplish the process of branching morphogensis and for the malignant counterpart of invasive growth, namely cell invasion and metastasis (Bardelli *et al.,* 1999, Khwaja *et al.,* 1998).

Branching morphogenesis is a complex differentiative process that requires modulation of gene expression. It has been found that epithelial tubulogenesis induced by SF/HGF requires the intervention of STAT, an unconventional transcriptional factor that can be directly activated by MET through an SH2 domain (Boccaccio *et al.,* 1998). Also the transcription factor NFkB, activated downstream in a transduction cascade initiated by MET, is required for branching morphogenesis (Muller *et al.,* 2002).

During the study of the complex signalling cascades orchestrated by MET, an intracellular transducer was discovered, named GAB1. This is likely to be a key co-ordinator of the cellular responses to MET (Maroun *et al.,* 1999, Weidner *et al.,* 1996). The significance of GAB1 for SF/HGF signalling is upheld by genetic evidence that GAB1 inactivation in mice phenocopies MET knock-out animals (Sachs *et al.,* 2000). GAB1 can be considered a scaffolding adaptor (such as the insulin receptor substrate, IRS), which does not contain a canonical phosphotyrosine interaction domain but is however recruited directly and indirectly by activated tyrosine kinases (Holgado-Madruga *et al.,* 1996). However, GAB1 features an extended phosphorylation only in response to SF/HGF stimulation, whereas a rapid and transitory phosphorylation is evoked by other growth factors. This may be a result of the low-affinity but high avidity association between MET and GABI through a specific *Met Binding Domain* (Gual *et al.,* 2000, Maroun *et al.,* 1999). Prolonged phosphorylation by MET could explain the ability of GAB1 to support the process of branching morphogenesis. In fact, GAB1 may function as a signal sustainer that enhances the recruitment and activation of transducers such as PLC-γ, the protein tyrosine phosphatase SHP2, PI3-K and the adaptor CRK-like (CRKL) (Gual *et al.,* 2000, Holgado-Madruga *et al.,* 1996). In particular, Shp2 and CRKL may lead to the sustained stimulation of the MAP kinase pathway, a feature which is required for the initiation and maintenance of branching morphogenesis(O'Brien *et al.,* 2004).

The complexity of the signalling leading to branching morphogenesis is further increased by recent studies which involve the presence of cell-surface molecules acting as MET co-receptors. The scatter factor receptors (MET and RON) can be activated by Plexins, when the latter are engaged by their ligands Semaphorins (Conrotto *et al.,* 2004, Giordano *et al.,* 2002).

Research has shown that within the physiological range of ligand concentration the wild-type MET receptor cannot efficiently engage signalling effectors and mediate invasive growth without being physically associated with the α6β4 integrin (Bertotti *et al.,* 2005, Trusolino *et al.,* 2001, and *et al.*, 2006). At the time of receptor activation by the ligand, the associated integrin is phosphorylated on tyrosines which provide supplementary docking sites for signal transducers (such as PI3-K, the Grb2 adaptor SHC, and the tyrosine phosphatase Shp2). The fact that the integrin functions as an adaptor contributes to the signalling strength and duration that is a distinctive feature of the branching morphogenesis programme.

When associated with MET, α6β4 integrin elicits intracellular signals independent of cell adhesion and acts solely as a biological amplifier of SF/HGF signalling (Trusolino *et al.,* 1998). The integrin requirement can be bypassed enforcing the signalling system by non-physiological high concentration of the ligand or by receptor over-expression (Chung *et al.,* 2004).

Besides α6β4 integrin, a variant of the CD44 transmembrane molecule has been found to associate to MET and to be involved in its activation and signalling (Orian-Rousseau *et al.,* 2002). CD44 has the ability to activate cell invasion but the mechanism underlying this activity was unclear. CD44 is a receptor for ECM components (such as hyaluronic acid, integrins and osteopontin) and with its intracellular moiety binds to the ezrin-radixin-moesin (ERM) family of proteins. The latter family of proteins control cell motility by mediating a mechanically powerful association of the actin cytoskeleton to the plasma membrane. Interestingly, ezrin has been identified as an effector of SF/HGF-dependent branching morphogenesis. In addition, deregulated expression of ERM proteins has been impli-

cated in cancer metastasis. Therefore, CD44 structurally and functionally couples SF/HGF receptor (and other tyrosine kinases) to the cytoskeleton and to the extracellular environment, thence localizing the motile activity of migrating cells to specific membrane sites (reviewed in Ponta *et al.,* 2003).

The genetic programme of branching morphogenesis

For branching morphogenesis to be accomplished, transcriptional and post-transcriptional regulation of gene expression is required, involving complex, differential gene expression over time. The *branching morphogenesis signature* is currently being investigated with the assistance of genomic and proteomic technologies. One of the genes more prominently upregulated by SF/HGF is osteopontin, an extracellular matrix-associated protein that binds integrins and CD44 cell-surface receptors (Medico *et al.,* 2001). It has been shown that osteopontin induction is critical for the morphogenic response to SF/HGF, and, interestingly, that expression of this protein is also associated with cancer invasive growth and metastasis (Medico *et al.,* 2001, Kang *et al.,* 2003).

It is likely that genes crucial for branching morphogenesis and invasive growth encode regulators of cell adhesion and cell migration through foreign environments.

In order to initiate ECM invasion epithelial cells must first disassemble adhesive structures that link them together and to the basement membrane. Intercellular adhesion is chiefly mediated by adherens junctions, where transmembrane E-cadherins are engaged in reciprocal homophilic recognition with their extracellular domain. With their intracellular moiety, E-cadherins bind to a submembraneous scaffold of cortical catenins that form a structural and functional bridge to the actin cytoskeleton (reviewed in Nagafuchi, 2001). The exact mechanism of adherens junction destabilization is still to be unravelled but at present it is known that it requires cadherin downregulation. In this process, a critical mechanistic role is played by Snail, a transcriptional repressor capable of switching off E-cadherin expression (Barrallo-Gimeno and Nieto, 2005). Although early reports suggested that the level of E-cadherin did not change during SF/HGF-induced scatter, a reduced expression of the cadherin protein in the same conditions has been reported by other groups and attributed to post-translational regulation (Khoury *et al.,* 2005, Miura *et al.,* 2001, Tannapfel *et al.,* 1994). Recently it has been shown that SF/HGF induces Snail activity, providing a mechanistic link with transcriptional regulation of E-cadherins (Grotegut *et al.,* 2006). Other reported effects of SF/HGF on E-cadherin include the redistribution of cadherin-catenin complexes from the actin cytoskeleton to the soluble membrane fraction (Balkovetz *et al.,* 1997, Balkovetz and Sambandam, 1999); the proteolytic cleavage of cadherins from the cell surface; and the destabilization of adherens junctions through tyrosine phosphorylation of cadherins and catenins (Herynk *et al.,* 2003, Shibamoto *et al.,* 1994).

The branching morphogenesis programme requires that, after mutual dissociation, epithelial cells cross the natural borders made up by the basement membrane to invade the surrounding stroma. This suggests the ability to react with previously unrecognised extracellular substrates through the engagement of appropriate integrins but also highlights the need to degrade the ECM by means of proteases. This is required so that a passage through macromolecules and cells can be opened and cryptic adhesion sites can be exposed.

Remodelling of integrin-mediated extracellular adhesion supplies both mechanic support for cell motility and an essential signal that protects cells from anoikis (apoptosis), a process elicited by failure in recognizing the extracellular environment (Frisch and Ruoslahti, 1997). SF/HGF controls the expression and activity of the entire integrin group thus allowing for recognition of the modified extracellular environment during cell invasion through multiple matrixes and tissue types, including basement membrane, connectives and endothelia. Indeed SF/HGF upregulates integrin transcription by way of continual activation of MAP kinases, at least in some cases (Liang and Chen, 2001, Nebe *et al.,* 1998), and stimulates integrin aggregation at specific adhesive sites thereby increasing their avidity for the substrate (Trusolino *et al.,* 1998, 2000).

SF/ HGF is also capable of regulating the expression and activity of key actors in ECM modelling, the matrix metalloproteases (MMPs, reviewed in Egeblad and Werb, 2002). MMPs manage to localize matrix digestion at the leading edge of migrating cells by interacting with integrins and CD44 (*see above*). SF/HGF has the ability to enhance the transcription of several MMPs and can induce conver-

sion of their inactive precursors into active enzymes (Balkovetz *et al.,* 2004, Rosenthal *et al.,* 1998). Somewhat unexpectedly though, recent findings have shown that broad-spectrum MMP inhibition does not hamper the initial phases of SF/HGF-triggered branching morphogenesis, featuring epithelial transition and matrix invasion. Instead, MMPs are necessary for terminal completion of tubulogenesis and reacquisition of the epithelial, polarized phenotype (O'Brien *et al.,* 2004).

It could be argued that MMPs are not limiting factors for cell invasion and that other ECM-degrading proteases, such as urokinase-type plasminogen activator (uPA) (Jeffers *et al.,* 1996, Pepper *et al.,* 1992), might have a predominant role in the branching morphogenesis programme. As previously mentioned, uPA is the main pro-HGF convertase and binds with high affinity inactive pro-HGF. Subsequently, by cleavage at a specific site, uPA transforms pro-HGF into a biologically active molecule (Naldini *et al.,* 1992). Moreover, SF/HGF can induce transcriptional upregulation of uPA expression, possibly sustaining a positive feedback on SF/HGF signalling (Boccaccio *et al.,* 1994, Pepper *et al.,* 1992).

As an SF/HGF effector, uPA is responsible for regulation of ECM degradation through conversion of plasminogen into plasmin, an enzyme active on a number of extracellular substrates. Plasmin proteolytic activity can be concentrated in proximity to the cell membrane, as its activator uPA binds to a cell surface receptor. Moreover the uPA receptor has further roles in the branching morphogenesis process as it also regulates cell adhesion (through binding of ECM substrates and modulation of integrin function), and evokes a signal transduction cascade inside the cell (reviewed in Sidenius and Blasi, 2003).

Lastly, SF/HGF utilises its pro-invasive capabilities not only to induce migration and survival of epithelial cells but also to modulate properties of the stromal microenvironment. Named as *landscaping effects*, these effects are indispensable for complex organogenesis during development, and can favour tumour growth and metastastization. The best known of these effects is the induction of angiogenesis through direct stimulation of endothelial cells (Bussolino *et al.,* 1992).

The control of MET expression

The MET promoter positively responds to a number of mitogenic stimuli, including growth factors, such as SF/HGF itself, tumour promoters (Boccaccio *et al.,* 1994, Gambarotta *et al.,* 1994) and activated oncogenes (Ivan *et al.,* 1997, Webb *et al.,* 1998). A prominent transcription factor for MET upregulation is ETS/AP1. Remarkably, ETS is activated by MET itself through the MAP kinase pathway and so offers an explanation as to why SF/HGF can induce its own receptor (Gambarotta *et al.,* 1996, Paumelle *et al.,* 2002). ETS concomitantly controls transcription of several genes essential for ECM regulation and thus for branching morphogenesis and invasive growth (Trojanowska, 2000).

A novel finding is that MET transcription is modulated by oxygen tension in tissues (Pennacchietti *et al.,* 2003), through a straightforward mechanism playing a leading role in regulating embryonic development and organ morphogenesis (Minet *et al.,* 2000). The cellular *oxygen sensor*, a protein called Prolyl-hydroxylase, regulates the availability of a transcriptional factor named hypoxia inducible factor-1α or HIF-1α (Semenza, 2003). When oxygen concentration lowers, for example as in tissues lacking adequate vascularisation, the oxygen sensor blocks the degradation of HIF-1α. As result, HIF-1α accumulates in the cell nucleus, and up-regulates the transcription of various genes including MET (Pennacchietti *et al.,* 2003). *In vitro* experiments have shown that hypoxia amplifies SF/HGF signalling and synergizes with SF/HGF in branching morphogenesis and invasive growth. MET overexpression is an absolute requirement for branching morphogenesis induced by hypoxia as shown by experiments of MET specific inhibition through RNA interference. Interestingly, analysis of human tumours has indicated that MET expression occurs at its highest level in hypoxic areas (in concomitance with elevated HIF-1α expression), while it decreases in proximity to blood vessels (where HIF-1α is barely detectable) (Pennacchietti *et al.,* 2003).

Interestingly, a further support to the key role of HIF-1α in MET transcriptional regulation derives from the fact that MET is over-expressed in tumours affected by inactivation of the Von Hippel-Lindau (VHL) tumour suppressor gene (Maranchie *et al.,* 2002). The VHL protein interacts with the oxygen sensor and targets HIF proteins for degradation in case of normal oxygen concentration. Inactivation of VHL prevents HIF-1α degradation even under nor-

moxic conditions, resulting in elevated MET transcription. In conclusion, hypoxia is a major driving force of MET expression *in vitro* and *in vivo*. Notably, this condition triggers not only expression of MET, but also of u-PA receptor (see above) (Rofstad *et al.,* 2002), and of the chemokine receptor CXCR4, which mobilizes normal stem cells and cancer cells (thus favouring metastasis) towards tissues that secrete the CXCR4-specific ligand SDF-1α (Muller *et al.,* 2001).

MET and stem cells

MET is thus an inducible gene that is highly sensitive to hypoxia and to extracellular stimuli controlling cell proliferation. Hypoxia and growth factors are elements present in the *stem cell niche*. This is the specific micro-environment responsible for modulating the properties of stem cells, including self-renewal, balance between symmetrical and asymmetrical duplication and mobilization. Much evidence suggests that in adult tissues MET expression may be restricted to the stem cell compartment and to its immediate progeny of progenitor and precursor cells. In the haemopoietic system, MET has been found in a small fraction of bone marrow cells, included in the subset expressing the CD34 marker, corresponding to haemopoietic progenitors and stem cells (Galimi *et al.,* 1994). The MET promoter invariably contains a binding element for the GATA family of transcription factors which are active in haemopoietic progenitors (Gambarotta *et al.,* 1994). MET, although expressed at low levels also by mature hepatocytes, is a hepatocyte stem cell marker, which can be used to positively select progenitors from differentiated cells with antibody-based cell sorting techniques (Suzuki *et al.,* 2002, Zheng and Taniguchi, 2003). In skeletal muscle, MET is expressed by myoblasts but it is downregulated at the time of differentiation in striated fibres (Anastasi *et al.,* 1997, Bladt *et al.,* 1995). Intriguingly, although not present in differentiated myofibers, MET is highly expressed in the skeletal muscle-derived tumours rhabdomyosarcomas (Ferracini *et al.,* 1996), indicating that neoplastic cells have regained MET expression or, more likely, that the tumour is derived from transformation of myogenic precursors.

Interestingly, MET is a transcriptional target for the β catenin/TCF transcription factor (Boon *et al.,* 2002), which is activated by the Wnt signalling pathway. This signalling cascade is physiologically switched on in gut stem cells and switched off during enterocyte differentiation. In the majority of colon cancers, the same pathway is aberrantly and constitutively operative; accordingly, MET is commonly found over-expressed in human colon carcinomas.

Based on all these assumptions, we can speculate that invasive growth evoked by SF/HGF is a natural genetic programme for stem cells. Interestingly, as stem cells multiply and circulate unrestrictedly to target and reach various locations in the organism (Wright *et al.,* 2001), they can be considered a physiological counterpart of metastatic cells. Therefore, the study of inappropriate activation of the invasive growth programme in normal stem cells can provide the key to understanding tumour progression towards metastasis (Boccaccio and Comoglio, 2006).

The role of the MET oncogene in tumour progression

MET was originally identified as a transforming oncogene generated by chromosomal translocation in an osteosarcoma cell line treated with a chemical carcinogen (TPR-MET) (Cooper *et al.,* 1984 Park *et al.,* 1986). The same translocation product has the ability to induce tumours in transgenic mice (Boccaccio *et al.,* 2005, Liang *et al.,* 1996), and is found in a small number of human gastric cancers (Soman *et al.,* 1990). However, activation of the MET oncogene is mostly achieved by different mechanisms in a large number of human tumours. The following mechanisms are usually involved in the constitutive activation of the MET tyrosine kinase: (a) point mutations causing activatory conformational changes in the catalytic site; (b) ligand-receptor autocrine circuits (which liberate cells from the requirement of paracrine SF/HGF supply), or increased paracrine stimulation; (c) MET overexpression, which favours heightened sensitivity to the factor or ligand-independent trans-activation.

Patient analysis has resulted in the strongest evidence that MET has a causal role in human cancers. A group of patients all affected by papillary renal carcinoma (HPRC), a hereditary form of cancer, showed germline missense mutations of MET (Olivero *et al.,* 1999, Schmidt *et al.,* 1998, Wahl *et al.,* 1999). The same mutations (and others) have also been found in non-hereditary tumours such as sporadic papillary renal cancer (Schmidt *et al.,* 1999, Wahl *et al.,* 1999) childhood hepatocellular

carcinoma (Park *et al.,* 1999), and gastric cancer (Lee *et al.,* 2000). Hereditary and sporadic papillary renal cancer is usually an indolent neoplasm, characterised by slow growth and local invasion (Danilkovitch-Miagkova and Zbar, 2002). However, somatic mutations of MET have been connected with increased aggressiveness of hepatocellular carcinoma and to the metastatic spread of head and neck squamous carcinoma (Di Renzo *et al.,* 2000). Notably, in this latter type of cancer the population of metastatic cells progressively enriches in MET expression, as the tumour invades the lymph-node stations stage by stage.

Intriguingly, in addition to genetic lesions, MET-based tumorigenesis might require abnormal SF/HGF stimulation (either through paracrine or autocrine mechanisms), as implicated by the fact that, in classical in vitro assays, cell transformation by MET mutants is possible only in the presence of its ligand, and is impaired when SF/HGF specific inhibitors are present (Michieli *et al.,* 1999).

A function for SF/HGF in maintaining MET-induced transformation has been identified in human tumours and assessed in mouse models. The SF/HGF autocrine loops and/or enhanced paracrine stimulation are observed in a wide range of cancers in patients, including osteosarcoma (Ferracini *et al.,* 1995, Scotlandi *et al.,* 1996), rabdomyosarcoma (Ferracini *et al.,* 1996, Scotlandi *et al.,* 1996), glioblastoma (Koochekpour *et al.,* 1997) and breast carcinoma (Tuck *et al.,* 1996, Yao *et al.,* 1996). This excessive autocrine/paracrine production of SF/HGF is often in association with aggressive tumour behaviour. Experimental induction of SF/MET autocrine loops in cell lines has proven to cause formation of invasive tumours after implantation in mice (Meiners *et al.,* 1998, Rong *et al.,* 1994). Transgenic mice expressing SF/HGF under a ubiquitous promoter develop a wide spectrum of neoplasms of both epithelial and mesenchymal origin (Takayama *et al.,* 1997). Among these tumours, melanomas exhibit a significant correlation between high metastatic potential and the presence of SF/MET (Otsuka *et al.,* 1998). In another mouse model, targeted expression of SF/HGF to the mouse mammary epithelium establishes autocrine and paracrine loops, sustaining formation of metastatic adenocarcinomas (Gallego *et al.,* 2003).

The most frequent mechanism of MET oncogene activation in human tumours is over-expression in the absence of any mutation of the coding sequence. Conceivably, over-expression is often due to hypoxia (see above), which is a frequent condition of tumours growing too rapidly to be adequately perfused by neo-angiogenic vessels. MET expression is, again, in association with the metastatic phenotype and with poor prognosis. For example, in colorectal carcinoma MET over-expression provides a selective advantage that fosters the tumours ability to produce lymph node and liver metastasis (Di Renzo *et al.,* 1995, Takeuchi *et al.,* 2003). In animal models, it has been shown that forced expression of wild-type MET in hepatocytes is sufficient to cause hepato-carcinomas, which regress after transgene inactivation (Wang *et al.,* 2001). Conceivably, increased expression of MET favours receptor dimerization and thus ligand-independent activation. However, the environmental availability of SF/HGF could always be mandatory. Therefore, *in vivo*, the tumour stroma, which physiologically produces, stores and regulates the activation of SF/HGF could have a critical landscaping role in promoting MET-dependent tumour growth, either in the presence of rare MET mutations, or in the commonly occurring situation of MET over-expression.

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Models of epithelial histogenesis

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Epithelial tissues emerge from coordinated sequences of cell renewal, specialization and assembly. Like corresponding immature tissues, adult epithelial tissues are provided by stem cells which are responsible for tissue homeostasis. Advances in epithelial histogenesis has permitted to clarify several aspects related to stem cell identification and dynamics and to understand how stem cells interact with their environment, the so-called stem cell niche. The development and maintenance of epithelial tissues involves epithelial-mesenchymal signalling pathways and cell-matrix interactions which control target nuclear factors and genes. The tooth germ is a prototype for such inductive tissue interactions and provides a powerful experimental system for the study of genetic pathways during development. Clonogenic epithelial cells isolated from developing as well mature epithelial tissues has been used to engineer epithelial tissue-equivalents, e.g. epidermal constructs, that are used in clinical practise and biomedical research. Information on molecular mechanisms which regulate epithelial histogenesis, including the role of specific growth/differentiation factors and cognate receptors, is essential to improve epithelial tissue engineering.

Key words: epithelial histogenesis – developmental biology – stem cell – differentiation – epithelial-mesenchymal interactions – tissue engineering.

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Epithelial histogenesis: a chance to unveil the face of human stem cells

Although epithelial cells are characterized by common structural features, especially their arrangement into cohesive sheets or thee-dimensional aggregates, they provide an enormous variety of biological functions, including protection, absorption, secretion, gametogenesis and special senses.

A major difference between developing and mature epithelial tissues is that differentiated epithelial cells that are not prone to locomotion. On the other hand, the migration of immature epithelial cells is essential for the development of many organs, including most of exocrine and endocrine glands, skin appendages and teeth. However, most of adult epithelial tissues are dynamic as to cell renewal and cell cycle activity. In fact, many processes that are observed during development are operating also in mature state, thus ensuring tissue homeostasis. Accordingly, most – if not all - of adult epithelial tissues are provided by specific stem cells and may be regarded to as useful models to study tissue formation and repair. The occurrence of stem cells in adult epithelial tissues has permitted the generation of bioengineered epithelial constructs that can be applied in cell and tissue therapy.

Epithelial stem cells, like other adult stem cells, are thought to be slow- or rarely-cycling cells, which retain clonogenicity and proliferative capacity for a long time. According to current models of tissue homeostasis, the division of a stem cell gives rise to another stem cell and one transient amplifying cell. Such a cell, after exhausting its proliferative potential, undergoes terminal differentiation, thus generating functional cells which are not further capable of proliferation (Leblond, 1981; Potten, 1983; Fuchs, 1990; Potten and Loeffler, 1990; Jones *et al.,* 1995). Since stem cells are slow cycling, they minimise DNA replication-related errors. Stemness properties are greatly conditioned by the microenvironment and positional creden-

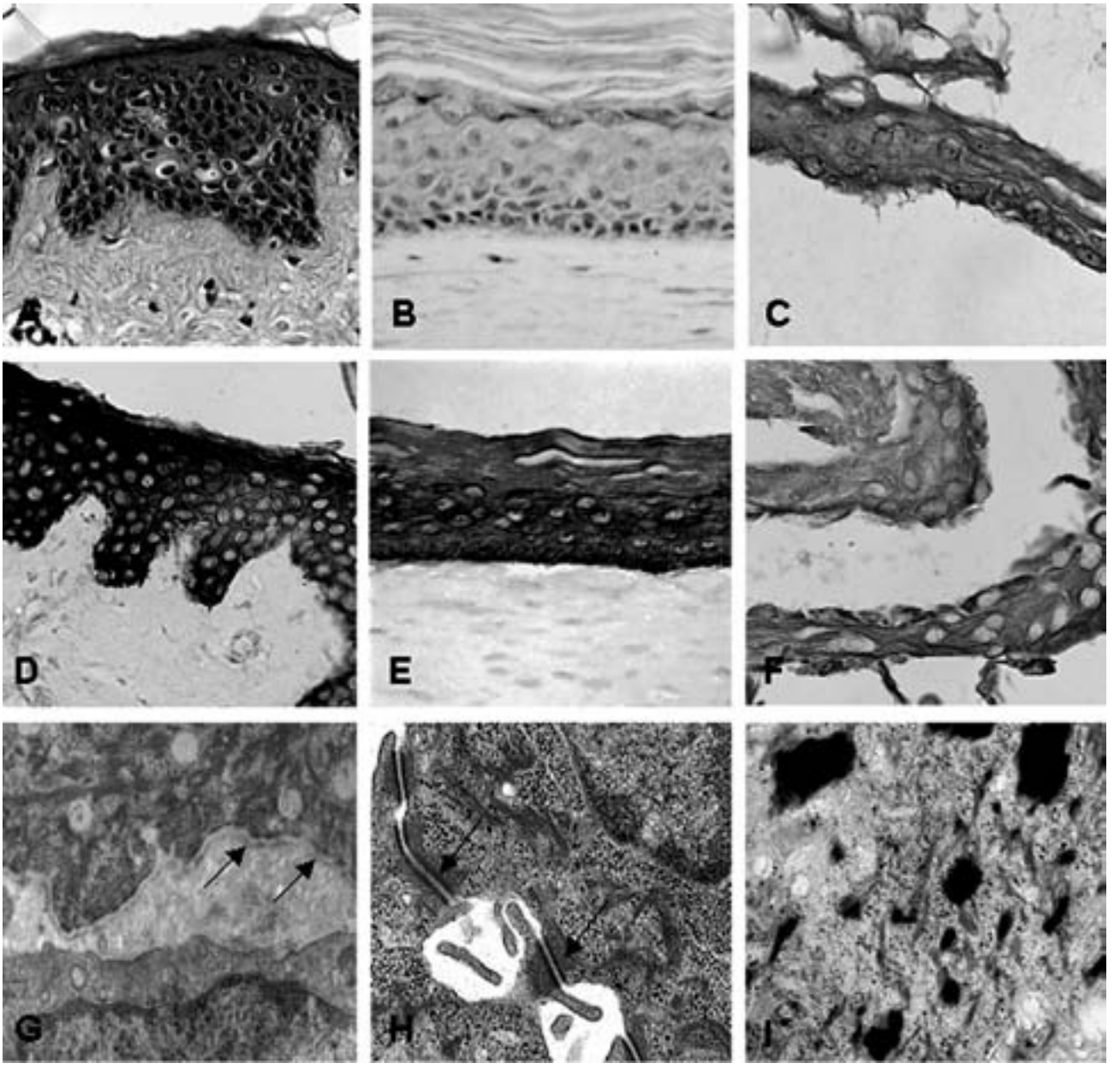

Figure 1. Aspects of histogenesis and cell differentiation in models of epidermis engineered *in vitro*. Tissue architecture of human natural skin (A), bilayered human skin equivalent (B), and simple epidermal construct (C). Cytokeratins are immunodetected thoroughout the cytoplasm of keratinocytes in natural skin (D), bioengineered skin (E), and epidermal construct (F), whereas connective tissue cells in the dermis (D) and dermal equivalent (E) are negative. In human skin equivalent, it is possible to observe a thin basement membrane at the dermo-epidermal junction (G), well-developed desmosomes between cells of the suprabasal layer (H) and irregular keratohyalin granules comparable to those observed in natural skin (I). Magn. x 300 (A,B,D,E), x 400 (C,F), x 20000 (G), x 26000 (H), x 18000 (I).

tials, thus suggesting the existence of a specific *niche* for each stem cells (Fuchs *et al.,* 2004). A major advantage of studying epithelial histogenesis is that stem cells are confined in discrete positions, e.g. the basal layer of stratified epithelia, thus making easier the identification of their niche compared to other tissues.

A challenge in stem cell research is the identification of molecular markers which allow the recognition of immature cell populations in tissues. Candidate markers for epithelial stem cells have been proposed which, correlated with cytokinetic parameters, have permitted the isolation and cloning of epithelial stem cells (Jones and Watt, 1993; Li *et al.,* 2004; Blanpain *et al.,* 2004).

Enamel epithelium: the histogenesis of the hardest tissue

The tooth germ represents a powerful model to understand molecular mechanisms of organogenesis which are mediated by epithelial-mesenchymal interactions. In fact, during odontogenesis, it is possible to monitor continuously cell differentiation in relative spatial positions, the production and secretion of specific molecules, and corresponding modifications of the extracellular matrix.

Mammalian teeth develop from two types of cells: stomodeal ectoderm cells, which form ameloblasts, and cranial neural-crest derived ectomesenchyme cells, which form pulp cells, odontoblasts and cementoblasts (reviewed by Sharpe, 2001; Cobourne and Sharpe, 2003). These two cells types interact to control the entire process of tooth initiation, morphogenesis and cytodifferentiation.

Epithelial-mesenchymal interactions that regulate the initiation of tooth formation, the differentiation of odontoblasts and ameloblasts and the acquisition of shape have been characterized by studies on tissue recombination (Kollar and Baird, 1968; Lumsden, 1988).

Furthermore, cell-to-cell and cell-matrix signalling pathways and related target nuclear factors have been identified as mediators of reciprocal communication between dental epithelial and mesenchymal cells (reviewed by Ruch, 1985; Slavkin, 1990; Jernval and Thesleff, 2000; Thesleff and Mikkola, 2002; Thesleff, 2003).

Tooth morphogenesis proceeds through characteristic stages, i.e. initiation, bud, cap and bell stages. As in many organs, the earliest evidence of tooth development is an epithelial thickening of the stomodeal lining epithelium. Under the instructive influence of the odontogenic mesenchyme, the inner enamel epithelium undergoes a precise developmental program, ultimately differentiates to the ameloblast phenotype and initiate the expression of tissue-specific enamel gene products which direct enamel biomineralization (Ruch, 1985; Jernval and Thesleff, 2000; Thesleff, 2003). The differentiation programme of the cells of the inner enamel epithelium can be summarized in three main phases, including pre-secretory, secretory and maturation phases (Warshawsky and Smith, 1974; Smith and Warshawsky, 1975; Nanci *et al.,* 1985; 1987; 1998). During pre-secretory stage, the cells of the inner enamel epithelium proliferate and acquire terminal cytodifferentiation; during the secretory stage, differentiated cells, which can be properly called ameloblasts, become functional and secrete specific enamel matrix components; in the maturation stage, ameloblasts are involved in the processing of enamel matrix which will result in the formation of the hardest tissue of human body. As in a romantic drama, ameloblasts, which are located at the surface of the tooth crown and have fulfilled their task, will die as soon as tooth erupts, after which time the enamel cannot be replaced by new synthesis.

The interaction of a cell with the surrounding extracellular matrix influences cell proliferation and differentiation gene expression via specific membrane receptors which activate downstream target genes. Much interest has been given to the phases of odontoblast and ameloblast differentiation which immediately precede the secretion of specific dentine and enamel matrices (Slavkin *et al.,* 1976; Bronckers *et al.,* 1993; Couwenhoven and Snead, 1994). It has been shown that expression of enamel specific genes is restricted to determined enamel epithelium cells that have withdrawn from the cell cycle and have undergone terminal differentiation to the ameloblast phenotype (Inai *et al.,* 1991; Casasco *et al.,* 1992, 1996).

Dentine and enamel specific proteins have been proposed as candidate regulatory molecules in dental epithelial-mesenchymal interactions. Indeed, it is possible to show that the secretion of enamel specific proteins immediately precedes dentine mineralization and that enamel proteins cross the basement membrane in the epithelial-mesenchymal interface (i.e. the future dentine-enamel junction) and reach the odontoblasts layer (Figure 2). A simplified scheme describing spatial and temporal aspects of odontoblast and ameloblast differentiation is shown in Figure 3.

The extension downward of cells of the enamel epithelium forms the so-called Hertwig root sheath which defines the final size of the tooth root, being later replaced by the cementum. Although it is generally believed that cementoblasts differentiate from dental follicle, which derive from cranial ectomesenchyme, it has also been suggested that cells of the Hertwig sheath may undergo epithelial-mesenchymal transformation and give rise to cementoblasts (reviewed by Bosshardt and Schroeder, 1996).

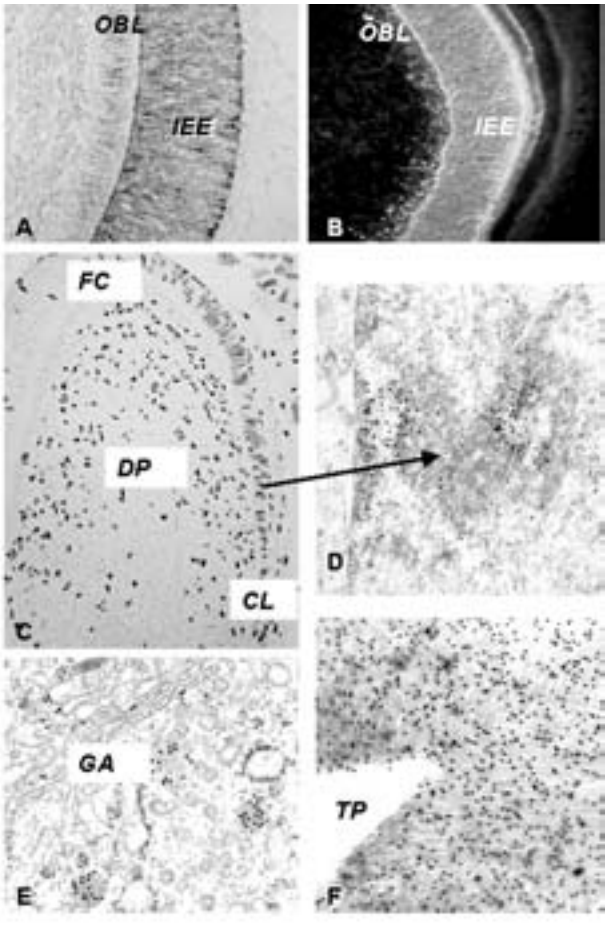

Figure 2. Aspects of histogenesis and cell differentiation in rat inner enamel epithelium. Intracytoplasmic localization of enamel matrix proteins (A) and 28 Kda-calciun binding protein (B) during early stage of ameloblast differentiation. C: Cell proliferation in tooth germ as observed by immudetection of brome-deoxyuridine: the number of positive cells in the inner enamel epithelium decreases from the cervical loop (CL) toward the forming cusp (FC). D: immunogold detection of brome-deoxyurdine within the nucleus of an immature cell of the inner enamel epithelium which is traversing the S phase of the cell cycle. E, F: immunogold detection of enamel matrix proteins within the cytoplasm of a secretory amelobast as well as the forming enamel. IEE, inner enamel epithelium; OBL, odontoblast layer; DP, dental pulp; GA, Golgi apparatus; TP, Tomes process. Magn. x 500 (A), x 400 (B), x 150 (C), x 25000 (D), x 40000 (E,F).

Enamel-related proteins secreted by epithelial cells of the Hertwig sheath are supposed to have an important role in cementogenesis during tooth development (discussed in Bosshardt and Nanci, 1998). Recently, clinical studies have demonstrated that the application of enamel proteins in bone defects around human teeth stimulates cementogenesis and new bone deposition, suggesting that regulatory molecules of odontogenesis may find a role in regenerative periodontal therapy (Gestrelius *et al.,* 2000).

Skin histogenesis: the story of the first bioengineered organ

In developing embryo, skin develops from the interaction of surface ectoderm and underlying mesenchyme. The primordium of the epidermis is a single layer of surface ectodermal cells. These cells proliferate and differentiate to form a layer of squamous epithelium, called periderm, and a basal germinative layer. Replacement of peridermal cells, which are part of the vernix caseosa, continues until about the 21st week; thereafter the periderm disappears and the stratum corneum forms.

In the adult, epidermis is a dynamic tissue in which terminally differentiated keratinocytes are replaced by the proliferation of new epithelial cells that undergo differentiation. Terminal differentiation of epidermal keratinocytes leads to the formation of the stratum corneum, which is not cellular but composed of intracytoplasmic remnants bound to the skin surface after the death of keratinocytes. Recent data support the view that keratinisation may be regarded to as a specialized form of apoptosis that produces the stratum corneum concomitant with keratinocyte cell death (Hathaway and Kuechle, 2002).

According to the spiral model of stemness proposed by Potten (1990), stem cell properties are lost gradually through successive rounds of division, whereas more committed progeny of epidermal stem cells undergoes an irreversible commitment to differentiation. Specific microenvironmental factors that regulate the growth and differentiation of keratinocyte progenitors remain poorly defined as well as unequivocal criteria for the identification of epidermal stem cells (Blanpain *et al.,* 2004; Fuchs *et al*., 2004; Tumbar *et al.,* 2004). Keratinocyte exhibit characteristic cytokeratin expression. In the epidermis, keratins 5 and 14 are expressed in the basal layer, while keratins 1 and 10 are found in the suprabasal layer. The transcription factor p63 has been proposed as a marker for keratinocyte stem cells (Pellegrini *et al.,* 2001; Koster and Roop, 2004; McKeon, 2004). Nevertheless, p63 is not restricted to stem cells, since it is expressed in all basal cells as well as a significant number of suprabasal cells. Interestingly, a combined identification of specific markers (e.g. transferrin receptor CD71 and α-6 integrin) has permitted the isolation of subpopulations of epidermal cells showing stemness properties (Jones and Watt, 1993; Li *et al.,* 2004).

Keratinocytes express several integrins, including

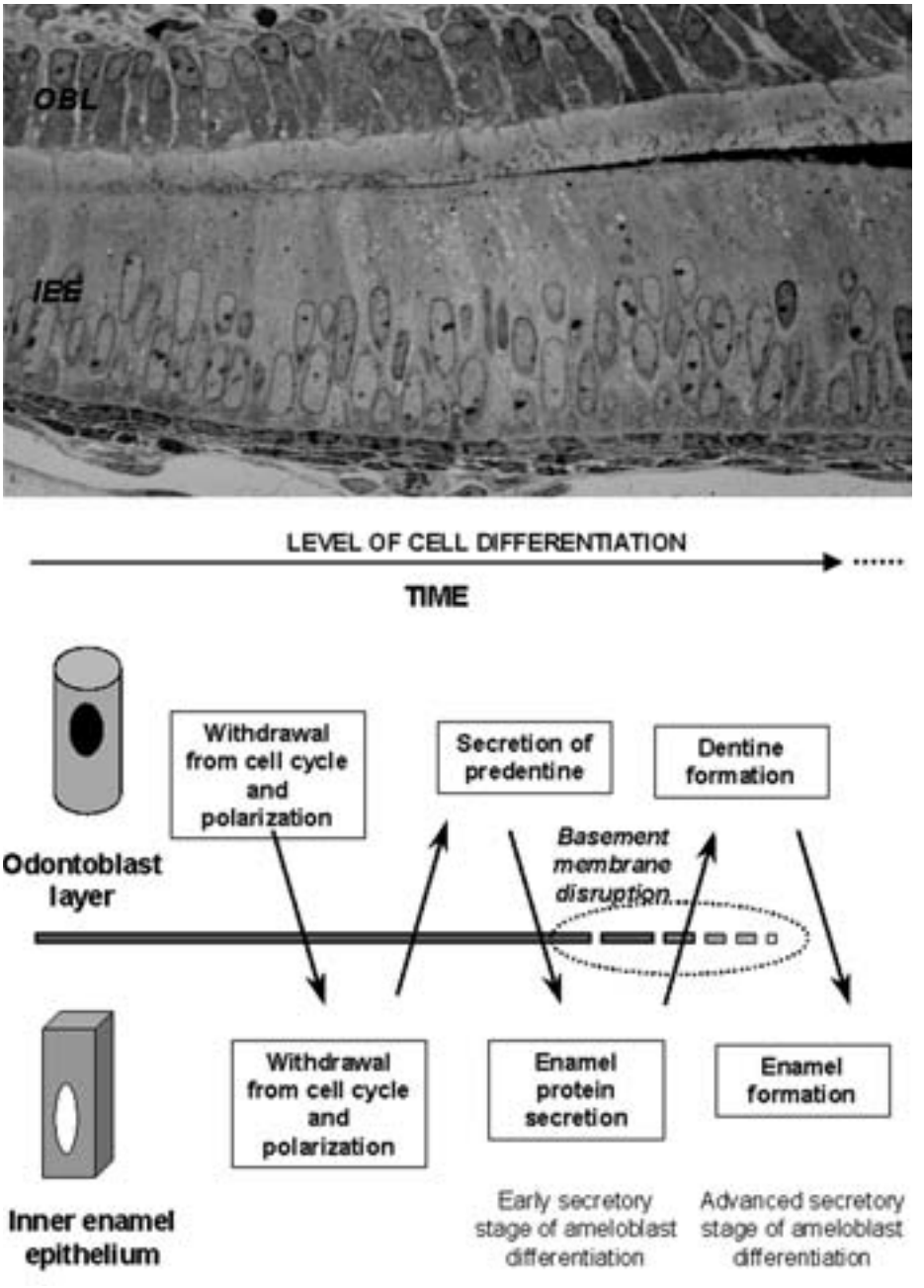

Figure 3. The microscopical picture shows the stages of the cells of the odontoblast layer (OBL) and of the inner enamel epithelium (IEE) which precede and go along with initial deposition of dentine and enamel. The scheme summarizes corresponding stages of cell differentiation and extracellular matrix maturation. Interestingly, the secretion of enamel specific proteins immediately precedes dentine mineralization and enamel proteins cross the basement membrane in the epithelial-mesenchymal interface (i.e. the future dentine-enamel junction) and reach the odontoblast layer. Ameloblasts withdraw from the cell cycle later that odontoblasts, as well as enamel formation is delayed compared to dentine formation. Magn. x 1000.

collagen-, laminin-, fibronectin- and vitronectin-receptors. It has been shown that integrins not only mediate adhesion to the underlying extracellular matrix, but also regulate keratinocyte differentiation (Watt *et al.,* 1993; Marchisio *et al.,* 1997); indeed detachment from the basement membrane seems to be a prerequisite to undergo terminal differentiation (Adams and Watt, 1990; Li *et al.,* 2004).

A major aim for tissue engineers is to develop new culture systems to change the way to conduct biological experiments and eliminate the *flat biology* of Petri dishes in favour of organotypic three-dimensional models. Recent advances in tissue engineering have permitted the generation of skin and epidermal substitutes *in vitro*. Different strategies have been conceived to engineer such substitutes and to date skin can be regarded to as the first bioengineered organ. Epidermal and dermal stem cells can be isolated from different sources, including developing and adult tissues. Long-term subcultivation of keratinocytes in vitro permitted the formation of epithelial layers similar to natural epidermis (Rheinwald and Green, 1975). Subsequently, epidermal constructs have been combined with dermal

equivalents to reconstruct the entire skin architecture (Bell *et al.,* 1981; Parenteau *et al.,* 1991; Stark *et al.,* 1999; Zacchi *et al.,* 1998). Indeed, organotypic co-culture made of keratinocytes and dermal cells have been shown to have many *in vivo*-like features, such as complete morphologic differentiation, assembly of a basement membrane, presence of cells with stem-like features, and epithelial-mesenchymal interactions (Casasco *et al.,* 2001a,b; 2004). Further experiments permitted the introduction of melanocytes, Langerhans cells, blood vessels and hairs in advanced models of artificial skin.

Tissue engineering experiments suggest that skin histogenesis is controlled by epithelial-mesenchymal interactions (Smola *et al.,* 1993). Although the precise mechanisms are largely hypothetical, several extracellular matrices of the dermo-epidermal junction and diffusible factors acting locally have been implicated in the regulation of keratinocyte growth and differentiation and skin homeostasis (Smola *et al.,* 1993; discussed in Turksen, 2005).

Future perspectives

Epithelial histogenesis involves dynamic patterns of multiple signalling cascades, and molecular and physical factors play their role with specific positional and time profiles, thus ensuring the regulation of cell proliferation, differentiation and functional assembly. If we understand how tissues develop, we might understand how engineer their artificial equivalents. The application of our knowledge in epithelial histogenesis has permitted the generation *in vitro* of tissue equivalents that are currently used in clinical practise (Gallico *et al.,* 1984; Falanga *et al.,* 1998) as well as high-fidelity models for quantitative research in biology and medicine, including tissue responses to drugs, genetic alterations, hypoxia and physical stimuli.

Moreover, information on the mechanisms that regulate epithelial histogenesis has been used to induce tissue regeneration in surgical procedures, according to biomimetics which derives principles from the nature for the design of innovative therapeutical strategies and tissue engineering systems. It is reasonable to believe that other factors which regulate the mechanisms of epithelial histogenesis will find biotechnological and clinical application where the need is to induce or enhance cell growth and differentiation.

Acknowledgements

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Adult stem cells: the real *root* into the embryo?

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During embryonic development, a pool of cells may become a reserve of undifferentiated cells, the *embryo-stolen adult stem cells* (ESASC). ESASC may be responsible for adult tissue homeostasis, as well as disease development. Transdifferentiation is a sort of reprogramming of ESASC from one germ layer-derived tissue towards another. Transdifferentiation has been described to take place from mesoderm to ectodermal- or endodermal-derived tissues and viceversa but not from ectodermal- to endodermal-derived tissues. We hypothesise that two different populations of ESASC could exist, the first ecto/mesoblast-committed and the second endo/mesoblast-committed. If confirmed, this hypothesis could lead to new studies on the molecular mechanisms of cell differentiation and to a better understanding of the pathogenesis of a number of diseases.

Key words: transdifferentiation, germ layers, self-renewing, gastrulation.

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Mechanisms responsible for tissue regeneration in organ homeostasis and recovery in adult organisms consist of 1) mitosis of differentiated functioning cells with a preserved proliferative activity (i.e. hepatocytes) and 2) generation of newly differentiated cell populations derived from adult stem cells (SC) (i.e. blood) (Abbott, 2006).

SC are defined as undifferentiated elements capable of self-renewal and differentiation into specialized cells. SC are considered resistant to toxic noxae and other damaging agents and have a great capacity to restore and renew (almost) all tissues of the human body. SC have been described in several tissues and it is not excluded that they could migrate to repair distant tissues. Indeed, they may be maintained in the systemic circulation and activated by pathophysiological stimuli from damaged tissues. Nevertheless, the regenerative activity of SC is not unlimited, because of senescence processes (Asahara and Kawamoto, 2004). Moreover, cancers may origin from SC, and also cancers' SC have the ability to self-renew, determining tumour resistance to therapy and recurrence (Abbott, 2006).

Nowadays, we are not really able to make a clear distinction between terms such as undifferentiated, stem-, germinal-, precursor- and progenitor- cells. Referring to embryonic tissues might be useful to disentangle ourselves in this terminological maze.

Embryo-stolen adult stem cells

The inner cell mass of the blastocyst gives rise to hypoblast and epiblast. The latter brings forth three germ layers, ectoderm, mesoderm and endoderm, that undergo proliferation through lineage commitment to form multi-, tri-, bi- and uni-potent undifferentiated cells, from which, finally, all differentiated cells and tissues of the body originate (Young and Black, 2004).

During embryonic development, a pool of cells may interrupt this continuum to become a reserve of

undifferentiated cells, that we may call *embryo-stolen adult stem cells* (ESASC). Whether ESASC are circulating or resident is a controversial topic, since the former does not exclude the latter.

Many evidences demonstrate the existence of lineage-committed and lineage-uncommitted SC in many adult organs *in vivo*, like bone marrow (Chiu, 2003), heart (Bellafiore *et al.,* 2006), kidney (Lange *et al.*, 2005), brain (Gritti *et al.*, 2002) and other organs. In each of these organs, these cells may originate one or more cytotypes. Examples include satellite myoblasts in skeletal muscle (Charge and Rudnicki, 2004), and hemangioblasts for hematopoietic and endothelial cells (Asahara and Kawamoto, 2004).

Moreover, isolation and characterization of SC *in vitro* becomes a practical approach to study these differentiation mechanisms. It is unfeasible and unpractical to report here all the plethora of studies that have been describing *in vitro* isolation of post-natal undifferentiated cells and experiments concerning their immunophenotyping, proliferation and differentiation potential and functional characteristics. Nevertheless, as anyone might observe, most of these studies describe SC derived from mesoderm differentiating towards ectodermal-derived tissues [i.e. from blood to neurons (Brazelton *et al.*, 2000)] or endodermal-derived tissues (i.e. from marrow to liver (Cantz *et al.*, 2004)), and SC isolated from ectoderm or endoderm originating mesodermal tissues [i.e. from neuron to blood (Bjornson *et al.*, 1999)].

The latter event, consisting of a sort of reprogramming of SC from one germ layer-derived tissue towards another, is called *transdifferentiation*. This theory is clashing with the common belief that once a SC is committed to a specific tissue lineage, it can not change its genetic program, reverting to a more primitive stage. Therefore, the existence of transdifferentiation *in vivo* is not universally recognised by scientific community. Young and Black (2004) for example suppose that transdifferentiation, when occurring *in vitro*, is due to contamination of isolated tissue by unrecognised progenitor cells.

At this time we would like to underline that none of the experiments of transdifferentiation conducted thus far have reported ectodermal-derived SC (EcDSC) or endodermal-derived SC (EnDSC) differentiating towards each other. To clarify this oddity, we should probably refer again to the embryo.

Gastrulation is probably an important step in ESASC formation

During the third week of embryonic development, gastrulation takes place from epiblast, from which all three germ layers derive. Gastrulation is a crucial time in the development of multicellular animals, since several essential steps are accomplished. Phylogenetically, gastrulation originates from two (diploblastic organisms, i.e. coelenterates) to three (all higher animals) layers. Whether the emergence of the mesoderm is linked to the evolution of axis formation in metazoan is not yet an assured fact (Technau and Scholz, 2003).

The molecular mechanisms responsible for gastrulation are still not well known. Also in triploblastic organisms, endoderm formation during gastrulation is not always linked to the formation of mesoderm, and different mechanisms have been proposed to explain this. Is commonly believed that some of the cells from the surface of the embryo move to the interior, replicating and thereby forming the new layers. These movements are coupled with the differentiation of the migrating cells (caused by the differential activity of certain genes) into histologically unique layers. The initial migration and differentiation of cells, which will then invaginate within the blastocoel, gives rise to the endoderm and mesoderm germ layers. In particular, in birds and mammals, epiblast cells converge at the midline and ingress at the primitive streak. Ingression of these cells results in formation of the mesoderm and replacement of some of the hypoblast cells to produce the definitive endoderm (Langman, 1995).

Moreover, since the ending *–derm* is usually referred to differentiated tissues, Technau and Scholz (2003) proposed to use the ending *–blast* to indicate proliferating but not yet differentiated tissues, such as germ layers (endoblast, ectoblast and mesoblast).

Hypothesis and conclusion

Since endoblast formation during gastrulation could be independent from the formation of mesoblast (Technau and Scholz, 2003), and since it is not well established whether endoblast development precedes mesoblast formation, one can not exclude the latter, as it should be phylogenetically intuitive. Consequently, ESASC could be considered as a pool of undifferentiated cells derived from a bi-layered (ecto-endoblast) embryo, that give rise to

the mesoblast and maintain multiple differentiative potentiality also in adult organism, being able to perform *transdifferentiation* from both EcDSC and EnDSC to mesoblast derived cells and vice versa; as a consequence, two populations of ESASC could exist, the first ecto/mesoblast-committed and the second endo/mesoblast-committed and this could explain why transdifferentiation from EcDC to EnDC has not yet been described. These two ESASC could be responsible for adult tissue homeostasis, as well as disease development, i.e. tumorigenesis.

In conclusion, in our opinion *in vitro* studies of SC could be imperative to discover the molecular mechanisms of cell commitment; the induction of cell differentiation *in vivo*, during the pathogenesis of a number of diseases, like Alzheimer, myocardial failure, celiac disease, etc, could become a new therapeutic target for the next generation of physicians.

Acknowledgement

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Extracellular matrix and growth factors in the pathogenesis of some craniofacial malformations

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The normal development of cranial primordia and orofacial structures involves fundamental processes in which growth, morphogenesis, and cell differentiation take place and interactions between extracellular matrix (ECM) components, growth factors and embryonic tissues are involved. Biochemical and molecular aspects of craniofacial development, such as the biological regulation of normal or premature cranial suture fusion, has just begun to be understood, thanks mainly to studies performed in the last decade. Several mutations has been identified in both syndromic and non-syndromic craniosynostosis patients throwing new light onto the etiology, classification and developmental pathology of these diseases. In the more common craniosynostosis syndromes and other skeletal growth disorders, the mutations were identified in the genes encoding fibroblast growth factor receptor types 1–3 (FGFR1, 2 and 3) where they are dominantly acting and affect specific and important protein binding domain. The unregulated FGF signaling during intramembranous ossification is associated to the Apert and Crouzon syndrome. The non syndromic cleft of the lip and/or palate (CLP) has a more complex genetic background if compared to craniosynostosis syndrome because of the number of involved genes and type of inheritance. Moreover, the influence of environmental factor makes difficult to clarify the primary causes of this malformation. ECM represents cell environment and results mainly composed by collagens, fibronectin, proteoglycans (PG) and hyaluronate (HA). Cooperative effects of ECM and growth factors regulate regional matrix production during the morphogenetic events, connective tissue remodelling and pathological states. In the present review we summarize the studies we performed in the last years to better clarify the role of ECM and growth factors in the etiology and pathogenesis of craniosynostosis and CLP diseases.

Key words: extracellular matrix; growth factors; craniosynostosis; cleft lip; cleft palate.

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During skull development, osteogenetic events lead to form the mesodermal neurocranium, which surrounds and protects the brain, and the neural crest-derived viscerocranium, which forms the face in mammals and supports the functions of feeding and breathing. The base of the neurocranium underlie the brain and is formed by endochondral ossification whereas the vault (calvaria) is formed by membranous ossification. The adjacent margins of membrane bones form the sutures which contain osteogenic stem cells and periosteal fibroblasts that differentiate into osteoblasts capable of producing new bone tissue, and are thus considered active sites of bone growth. Growth and expansion of the skull vault takes place to allow free growth of the brain. Craniosynostosis arises when this mechanism fails, because of the premature loss and ossification of sutural growth centres (Morriss-Kay and Wilkie, 2005). The normal development of the upper jaw and of the palate starts at about the 6th week of intra-uterine life and requires growth and fusion of the medial nasal processes and maxillary processes to form the lip, while the fusion of the palatal shelves to form the secondary palate occurs later (10th week). Craniofacial malformations and in particular orofacial clefting are the most common birth defects that occur in humans. Clefts of the lip, with or without cleft palate, and those that involve the palate only, are due to a failure in fusion of the facial processes and/or palatal shelves, and constitute two forms of oral-facial clefts considered separate birth defects involving many (but not all) of the same genetic and environmental causes (Carinci *et al.,* 2003).

Craniosynostosis (Apert and Crouzon syndromes)

Sutures contain osteogenic stem cells as a reservoir of potential new osteoblasts and are thus active

sites of bone growth. The premature fusion of one or more skull sutures due to altered osteogenic processes at the time of calvarian development is the most severe anomaly of the calvarium and is known as craniosynostosis, which prevents further bone growth along the edges. This leads the cranial vault to expand in other directions, thereby giving rise to a wide variety of pathological phenotypes. Crouzon syndrome accounts for 5% while Apert syndrome accounts for 4-5% of all cases of craniosynostosis (about 343 per million newborns; Cohen and MacLean, 2000).

Comparative studies indicate that although Apert and Crouzon syndromes present very similar cranial anomalies, they differ in cranial development (Kreiborg *et al.,* 1993).

Pathogenesis of craniosynostosis

Until just over a decade ago, little was known about the causes of craniosynostosis. Since then, several mutations were identified in both syndromic and non-syndromic patients throwing new light onto the etiology, classification and developmental pathology of these diseases (Morriss-Kay *et al.,* 2005).

The first mutation to be identified was a heterozygous missense mutation in homeotic MSX2 gene in patients with craniosynostosis type 2, also known as Boston-type, a rare syndrome confined to a single large family. TWIST1 and EFNB1 are also two significant genes (Wilkie, 2006). In other more common craniosynostosis syndromes and other skeletal growth disorders, the mutations were identified in the genes encoding fibroblast growth factor receptors (FGFRs). FGF2, a member of the FGF family, binds to high- and low-affinity receptors that are four different transmembrane tyrosine kinase receptors (FGFR1, -R2, -R3, and -R4). Upon FGF2/FGFR binding, the FGFR2 receptors dimerise and thus activate the intracellular tyrosine kinase domains. This is followed by phosphorylation of cellular proteins and transmission of signals into the cell that initiate a cascade of signals influencing cell division and differentiation. Membranous ossification of the skull vault is characterized by expression of FGFR genes in preosteoblasts and osteoblasts (Delezoide *et al.,* 1998). Genetic analysis of many human skeletal disorders have demonstrated the critical role of the FGF-FGFR system in endochondral and endomembranous ossification. In particular, defective or excessive fibroblast growth factor (FGF) signaling interferes with normal cranial suture morphogenesis (Naski *et al.,* 1998). Activating missense mutations occurring in FGFR2 (Kan *et al.,* 2002) cause an unregulated FGF signaling during intramembranous ossification and are associated to an important category of craniosynostosis as Apert, Crouzon and Pfeiffer (MIM 101600) syndromes (Pfeiffer syndrome is caused by mutations in either the FGFR1 or FGFR2 gene). In particular, the most common mutation of FGFR2 in Crouzon syndrome is C342Y generated by the Cys342-to-Tyr substitution. In Apert syndrome, the two adjacent amino acid substitutions Ser252-to-Trp (S252W), and Pro253-to-Arg (P253R) in the linker stretch between the second and third Ig-like domains, account for the vast majority of cases of syndrome (Robertson *et al.,* 1998) with frequencies of 71 and 26% respectively for the S252W and the P253R mutations. The C342Y mutation results in a constitutive (ligand-independent) receptor activation (phosphorylation) and causes excessive signalling (Ibrahimi *et al.,* 2004). Apert S252W and P253R mutations and some Pfeiffer mutations activate receptor only in the presence of ligand (ligand-dependent) (Yu *et al.,* 2000).

It is noteworthy that equivalent mutations in FGFR1, FGFR2, and FGFR3 genes result in different phenotypes: the P252R FGFR1 mutation was identified in a mild form of Pfeiffer syndrome, the P253R FGFR2 mutation causes Apert syndrome, and the P250R mutation of FGFR3 causes Muenke syndrome. An interesting hypothesis to explain such heterogeneity of phenotypes with similar mutations is that they function interactively, and loss- or gain-of-function mutations in one gene that affect the function of the protein may have secondary effects on one or both of the other FGFRs (Morriss-Kay *et al.,* 2005).

Cleft of the lip and/or palate

Cleft of the lip and/or palate (CLP) is the most common congenital orofacial malformation in humans. The disease is multifactorial and is probably caused by genetic and/or environmental factors (Carinci *et al.,* 1995; Scapoli *et al.,* 1997, 1998; Pezzetti *et al.,* 1998). Although a putative role of ECM has been assumed for long time in the genesis of CLP, few reports have analyzed the composition and relative amount of different types of glycosaminoglycans (GAG) and collagen in human cell

culture either under normal conditions or during stimulation with growth factors or clefting drugs. Interaction between ECM and cytokines is thought to be crucial for palatal development. Indeed, control of ECM metabolism in the embryonic orofacial region seems to be essential for normal palatal development. ECM molecules, in turn, promote the activities of growth factors and cytokines present in epithelial cells and palatal mesenchyme (Qiu *et al.,* 1995). The localization of transforming growth factors (TGFα and TGFβ isoforms) and fibroblast growth factors (FGF) in craniofacial tissues suggests that they carry out multiple functions during development.

CLP can be subdivided into syndromic (such as chromosomal, Mendelian, teratogen-based, and uncategorized syndromes) and non syndromic forms (about 70% of cases). Occurrence estimates range between 1/300 and 1/2500 births for cleft lip with or without cleft palate (CLP) and around 1/1500 births for cleft palate alone (CP). The non syndromic CLP arises when nasal processes and/or palatal shelves fail to fuse because genetic abnormalities and/or a perturbed environment alter extracellular matrix (ECM) composition and affect cell patterning, migration, proliferation and differentiation (Young *et al.,* 2000).

Pathogenesis of CLP

The non-syndromic CLP have a more complex genetic background if compared to craniosynostosis syndrome because of the number of involved genes and type of inheritance (for review: Carinci *et al.,* 2003). Some genes are involved in the formation of CLP defects. Among them, transforming growth factor-β3 (TGF-β3), whose mutations and/or deficiences give rise to cleft palate in humans (Lidral *et al.,* 1998) and mice (Kaartinen *et al.,* 1995); retinoic acid receptor-α (RARA), even though opinions are divergent as to whether RARA should be considered as a candidate gene in CLP (Scapoli *et al.*, 2002; Stein, 1995); γ-aminobutyric acid type A receptor β3 (GABRB3), a protein receptor for γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system, is also probably involved in human CLP malformation (Scapoli *et al.,* 2002; Baroni *et al.,* 2006). Moreover, the influence of environmental factor (cigarette smoking, alcohol consumption, corticosteroid, retinoic acid and anticonvulsant taken during the gestation period) on CLP onset, makes difficult to clarify the primary causes of this malformation.

Morphogenetic signals of ECM components during craniofacial development

During osteogenesis, mesenchymal cells differentiate in osteoblast lineage and produce a mineralized ECM that takes control of morphogenetic events. The ECM complex is formed by proteoglycans (PG), glycosaminoglycans (GAG), fibronectin, collagens, and other glycoproteins, which are differently distributed and organised in tissues and stages of development. The ECM-cell receptor-link transmits signals across the cell membrane in the cytoplasm, thereby initiating a cascade of events that culminates in the expression of specific genes. Fibronectin interacts with fibrils of type I collagen (Moursi *et al.,* 1996), another ECM molecule that realizes a matrix-mediated tissue interaction. Laminin, fibronectin, collagen type I and IV are all distributed in characteristic maps in epithelial-mesenchymal interfaces involved in the formation of avian embryo cartilaginous neurocranium (Thorogood *et al.,* 1988). Other ECM components such as PG, and particularly syndecan-III and biglycan (Xu *et al.,* 1998), control spatially and temporally ossification during fetal development and play a role in the terminal differentiation of embryonic cells.

In addition, an important role in the craniofacial morphogenesis is ascribed to matrix metalloproteinases, enzymes involved in ECM degradation. All these different transient patterns of ECM components are interpreted as reflecting different levels of morphogenetic specification of skull form in the developing head.

ECM in pathological conditions

Alterations of the normal balance between ECM synthesis and catabolism have to be considered a relevant factor in establishing connective diseases (Carinci *et al.,* 2005; Malemud *et al.,* 2006).

Potential defects in connective disease may thus be depending on one or more of the following event sequence: ECM synthesis and catabolism, ECM control on growth factor activity, stimulus by growth factor on specific receptor, intracellular message production, effect on ECM macromolecules synthesis.

Role of growth factors in skull morphogenesis and osteogenesis

A significant role in skull morphogenetic events is played by signal molecules present in the early embryo *in vivo*. Interleukins (ILs), transforming growth factor β (TGF-β) and fibroblast growth factor2 (FGF-2) are putative *signal peptides* present in skull tissues at the time of active differentiation and morphogenesis. Interleukin 1 (IL-1) and interleukin 6 (IL-6) are two of other cytokines that are involved in the regulation of bone cell functions. IL-1 stimulates cell replication and, at a low dose, bone collagen synthesis (Canalis *et al.,* 1989); IL-6 enhances bone turnover, stimulating bone resorption processes. TGF-β and bone morphogenetic proteins (BMPs), a subfamily of the TGF-β protein family, regulate chondrocyte proliferation and differentiation, and the ossification of endochondral bones. Disturbed TGF-β signaling lead to a variety of human skeletal disorders.

FGF2, which is the most abundant growth factor in the vault, is another regulator of bone development. FGF2/FGFR binding induces a broad spectrum of activities into the cells, including growth and cell migration increase and induction of proteases such as plasminogen activator, catepsin B, kallicrein (Szebenyi and Fallon *et al.,* 1999).

Materials and Methods

We performed studies in cells obtained from Apert and Crouzon patients during corrective surgery for the malformations. Fibroblasts were obtained from the galea-pericranium and osteoblasts from the parietal bone near the coronal suture. CLP fibroblasts were obtained from the oral flap edge of hard secondary palate subjects with familial non-syndromic cleft lip and palate and from age-matched controls. All cells were obtained during corrective surgery for the malformation. Human tissues were obtained with a protocol approved by our institutions. Informed consent was obtained from all parents after the nature of the study had been fully explained.

To study GAG neosynthesis, confluent cultures were labeled with 5 pCi/mL of (3H)-glucosamine hydrochloride (NEN Du Pont de Nemours, RFG; s.a. 29 Ci/mmol) and labelled GAG were precipitated from recovered supernatants and cells.

Secreted collagens were analysed in confluent cultures, added with L-ascorbic acid (50 pg/mL), (3-aminopropionitrile fumarate (50 μ/mL) and 10 μCi/mL of (3H)proline (Du Pont NEN, s.a. 29 Ci/mmol).

Fibronectin was isolated from media of confluent cultures labeled with 20 pCi/mL (35S)-methionine (s.a.>1,000 Ci/mmol, Amersham International, England, U.K.) during the last 3 hours of incubation. Samples were subjected to SDS-PAGE analysis, followed by densitometric quantification of fluorographs.

Gene expression was analysed by RT-PCR and measured semiquantitative radioactive RT-PCR and/or Northern blotting and autoradiography; for FGF2 and TGF β receptor counting, a radioactive binding assay was used.

Expression gene profiling was performed after total RNA extraction from cultured normal and pathologic cells. cDNA was synthesized by using Superscript II (Invitrogen, Paisley, England, UK) and amino-allyl dUTP (Sigma, St. Louis, MO, USA). Human 19.2K DNA microarrays, containing 19,200 expressed sequence tag (ESTs), corresponding to at least 15,448 different Unigene clusters, were used (Ontario Cancer Institute, Ontario, Canada). Hybridized slides were scanned using GenePix 4000A (Axon Instruments, Foster City, CA, USA). Images were analyzed by GENEPIX PRO 3.0 (Axon Instruments) and data extracted as described (Carinci *et al.,* 2002). Genetic analyses were conducted using a sample composed by 38 multiplex pedigrees and 200 sporadic patients. Polymorphic DNA markers, mainly microsatellite repeats, were typed by PCR using fluorescent-labelled primers and subsequent electrophoresis on a DNA sequencing equipment. Parametric linkage analyses with Lod score method were performed by LINKAGE software package. Non parametric analyses were performed with the standard transmission disequilibrium test (TDT).

Results

Apert syndrome

Periosteal pericranial fibroblasts from Apert patients synthesized and secreted greater amounts of sulphated (heparan sulphate, HS; chondoitin sulphate, CS; dermatan sulphate, DS) and non-sulphated (hyaluronic acid, HA) GAG compared to normal cells (Bodo *et al.,* 1997). Treatment with IL-1 and IL-6 reduced HA secretion by Apert cells. IL-1 significantly increased CS secretion by Apert fibroblasts and IL-6 enhanced HS and DS secre-

tion. These results demonstrated the abnormal phenotype in Apert fibroblasts and a possible involvement of IL1 and IL6 in the pathophysiology of the malformation. Apert fibroblasts secreted less IL-1 and IL-6 than normal cells (Bodo *et al.,* 1998a), whereas IL-1 receptor antagonist (IL-1 ra, which binds to IL-1 receptors in competition with IL-1 but does not elicit intracellular response from this binding), was markedly more secreted. The active transforming growth factor-β1 (TGF-β1), an IL-1 antagonist, was less secreted in Apert than in normal cells. The observed imbalance in the production of ILs suggests that ILs could be the natural autocrine regulators of ECM production in Apert fibroblasts. Since TGF-β1 is able to modulate ECM macromolecule accumulation in fibroblasts (Bodo *et al.,* 1999b), and a variety of osteoblast activities (Janssens *et al.,* 2005), we also analysed its expression and secretion which resulted both increased in Apert osteoblasts *in vitro* (Locci *et al.,* 1999). Moreover, the level of TGF-β1 was decreased by the addition of FGF2. All these results lead to hypothesise that *in vitro* differences between normal and Apert osteoblast phenotype may correlate to different TGF-β1 cascade patterns, probably due to an altered balance between TGF-β1 and FGF2.

In a recent report (Baroni *et al.,* 2005), we studied the ECM matrix and the FGF2 effects in primary cultures of Apert osteoblasts carrying the FGFR2 P253R mutation, to test the hypothesis that the mutation in FGFR2 domains is associated with a different osteoblastic differentiative phenotype and so obtain a clearer understanding of the mechanisms involved in matrix-mediated altered cranial differentiation. We evaluated gene expression of osteocalcin, a marker of the mature osteoblasts and for the first time, the expression of runt-related transcription factor-2 (RUNX2), a factor required for early commitment of mesenchymal precursor cells into osteoblasts (Ducy *et al.,* 2000). Compared with wild-type controls, osteocalcin mRNA was down-regulated in Apert osteoblasts, and RUNX2 mRNA was differentially spliced (Figure 1). Total protein synthesis, fibronectin, type I collagen and FGF2 secretion were up-regulated, confirming their modified phenotype, while protease and glycosidase activities and matrix metalloproteinase-13 (MMP-13) transcription were decreased, suggesting an altered ECM turnover. High affinity FGF2 receptors were studied and they resulted up-regulated in Apert osteoblasts. Analysis

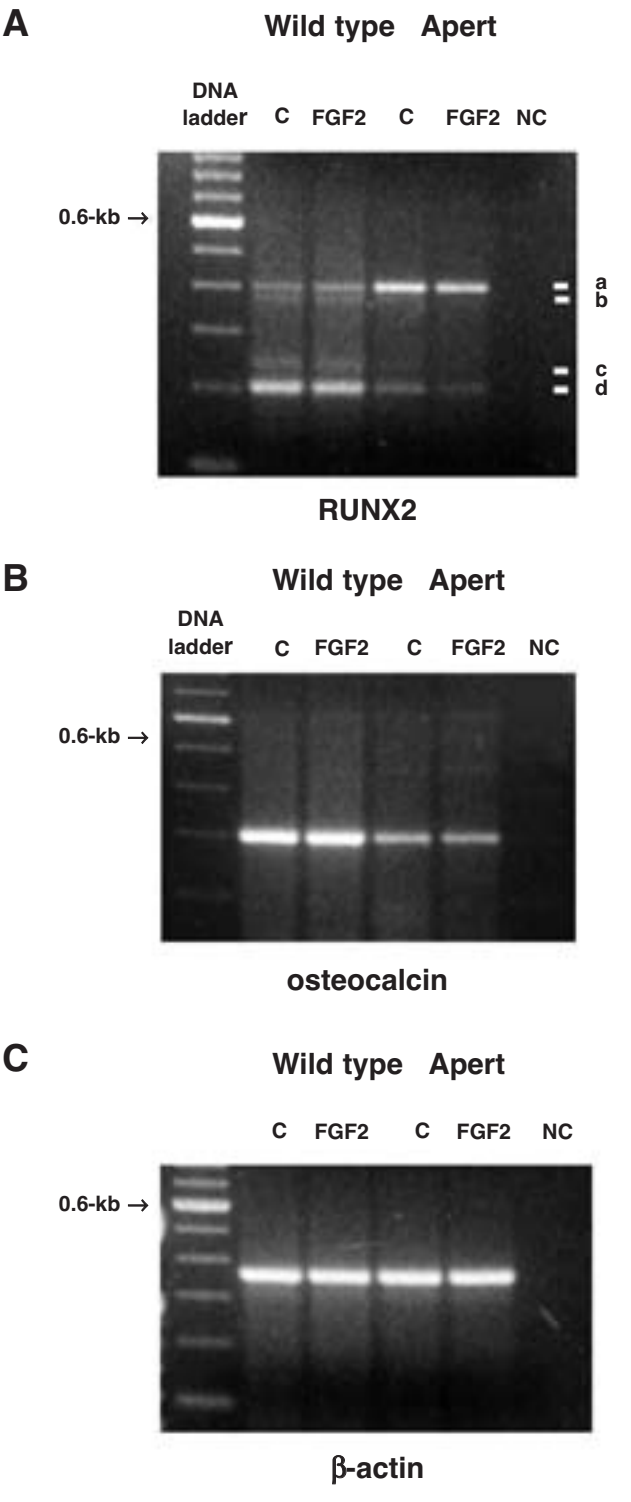

Figure 1. RT PCR analysis of RUNX2 and osteocalcin mRNAs. Panel A. The a, b, c, and d bands represent forms of differentially spliced RUNX2 mRNAs. The product sizes for osteocalcin (panel B) and β-actin (panel C) are 304-bp and 351-bp respectively. The lane marked NC represents the negative control of PCR, in which cDNA was omitted. The figure shows a photograph of the gel representative of one experiment performed on the cells from one Apert patient and from the respective control. Similar results were seen in the other two separate experiments, relatively to the other two patients and the respective controls. Figure (from Baroni, 2005) reproduced with the kind permission of © Wiley-Liss, Inc.

Table 1. Differentially expressed genes in craniosynostosis vs. wild type cells: ESTs up-regulated in craniosynostosis cells (down in wild type cells).

Name	*Symbol*	*GOabr*
3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble)	HMGCS1	Cytoplasm | soluble fraction | lipid metabolism | hydroxymethylglutaryl-CoA synthase
AD024 protein	AD024	
Aldehyde dehydrogenase 3 family, member B1	ALDH3B1	Lipid metabolism | alcohol metabolism | aldehyde dehydrogenase
Amiloride binding protein 1 (amine oxidase [copper-containing])	ABP1	Metabolism | peroxisome | amine oxidase | drug binding
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	ATP5J2	
Breast carcinoma amplified sequence 2	BCAS2	Spliceosome | RNA processing | pre-mRNA splicing factor
CDC37 cell division cycle 37 homolog (S. cerevisiae)	CDC37	Chaperone | protein binding | protein targeting | cell cycle regulator | regulation of CDK activity
Cell death-inducing DFFA-like effector b	CIDEB	Induction of apoptosis by DNA damage
CGI-127 protein	LOC51646	
Chromosome 21 open reading frame 80	C21orf80	
Crystallin, alpha B muscle contraction	CRYAB	Vision | nucleus | cytoplasm | chaperone | protein folding |
Cyclin D-type binding-protein 1	CCNDBP1	
Cytochrome c oxidase subunit VIc	COX6C	Energy pathways
Dipeptidylpeptidase VI	DPP6	Dipeptidyl-peptidase

A selection of genes with significant t-test values is reported in this table. Table (from Carinci, 2002) reproduced with the kind permission of The Feinstein Institute for Medical Research.

of signal transduction showed elevated levels of Grb2 tyrosine phosphorylation and the Grb2-p85 beta association, which FGF2 stimulation strongly reduced. All together these findings suggest increased constitutive receptor activity in Apert mutant osteoblasts and an autocrine loop involving the FGF2 pathway in modulation of Apert osteoblast behavior.

In conclusion, our results show that the FGFR2 Apert mutation lead the FGF2/FGFR receptor system to a gain of function and it seems to influence osteogenic phenotype in our primary culture cell system. Our results agree with other reports (Wilkie, 2005).

Crouzon syndrome

In 2002, we performed a genetic profile by DNA microarrays in patients with Apert or Crouzon syndromes (Carinci, 2002). The experiment yielded different clusters of expressed sequence tags (ESTs). Expression profiles from craniosynostosis-derived fibroblasts differed from those of wild-type fibroblasts (Table 1). Furthermore, two EST clusters discriminated the Crouzon from Apert fibroblasts (Table 2). The differentially expressed genes covered a broad range of functional activities, including bone differentiation, cell-cycle regulation, apoptotic stimulation, and signaling transduction, cytoskeleton, and vescicular transport. So we concluded that the transcriptional program of craniosynostosis fibroblasts differs from that of wild-type fibroblasts, and moreover, the expression profiles of Crouzon and Apert fibroblasts can be distinguished by two EST expression clusters. The different expression profiles in craniosynostosis cells and wild-type cells supports the hypothesis that craniosynostosis cells present either a different degree of constitutive activation or lower levels of mutated FGFR with a negative feed-back loop propagating downstream effectors. In other words, the abnormal receptor conformation could mimic and thus accentuate the effects of FGF2-FGFR binding or, on the contrary, reduce the levels of binding. In both scenarios, bone differentiation would be abnormal.

The intriguing difference between the two Crouzon and the Apert patients reported in that paper for the first time suggests that the syndromes

Table 2. Differentially expressed genes in Crouzon vs. Apert fibroblasts: ESTs up-regulated in Crouzon fibroblasts.

CloneID	*Name*	*Cytoband*	*Goabr*
41653	Fatty acid desaturase 1	11q12.2-q13.1	C-5 sterol desaturase | fatty acid desaturation | integral membrane protein
28193	Ubiquitin specific protease 28	11q23	
502965	KIAA1391 protein	11q23.2	
279904	Enhancer of invasion 10	14q11.1	
154147	Golgi associated, gamma adaptin ear-containing, ARF binding protein 2	16p12	
469423	LIS1-interacting protein NUDE1, rat homolog	16p13.11	
114341	KIAA1321 protein	17q11.1	
40462	Zinc finger protein 264	19q13.4	
171569	Janus kinase 1 (a protein tyrosine kinase)	1p32.3-p31.3	Protein phosphorylation | protein tyrosine kinase | intracellular signaling cascade | peripheral plasma membrane
429621	Hypothetical protein FLJ23231	1p35.2	
178037	RNA, U17D small nucleolar	1p36.1	
194512	PC326 protein	1q23.2	
172447	Chromosome 20 open reading frame 154	20p12.3	
203939	RNA-binding region (RNP1, RRM) containing 2	20q11.21	Nucleoplasm | RNA processing | pre-mRNA splicing factor
152378	Hypothetical protein FLJ20635	22q13	
381382	Hypothetical protein	22q13.2	
112048	Prenylcysteine lyase	2p13.3	
117322	Eukaryotic translation initiation factor 4E-like 3	2q36.1	mRNA cap binding | translation factor | translational regulation
156984	Transketolase (Wernicke-Korsakoff syndrome)	3p14.3	Transketolase
124459	Chemokine binding protein 2	3p21.3	Chemotaxis | immune response | plasma membrane | chemokine receptor | developmental processes | integral plasma membrane protein | G-protein linked receptor protein signalling pathway
143261	KIAA1160 protein	3q22.1	

Cluster of ESTs corresponding to the Crouzon fibroblasts. These ESTs are significantly modulated (p<0.01) in Crouzon fibroblasts when compared to Apert and wild type fibroblasts. Fifty nine ESTs and twenty one ESTs are up-regulated in Crouzon fibroblasts. The IMAGE clone ID, attributes, cytoband and Gene Ontology annotation when available are shown. Table (from Carinci, 2002) reproduced with the kind permission of The Feinstein Institute for Medical Research.

might be linked to different genetic backgrounds and might explain how identical FGFR mutations are associated with different clinical features.

We studied the phenotypes of normal and Crouzon fibroblasts and osteoblasts together with the effects of FGF2 on the gene expression of some ECM proteins (Bodo *et al.,* 1999a). Spontaneous or FGF2-modulated release of ILs was also assayed. When we analysed the role of FGF2 in the expression of ECM macromolecules in a cellular model constituted by osteoblasts from Crouzon patients, we found that the growth factor induced changes in the GAG profile and in the levels of PG and procollagen alpha1 (I) mRNAs and downregulated heparan sulfate GAG chains. Moreover, FGF2-induced IL secretion differed in normal and Crouzon osteoblasts. These studies provide evidence that FGF2 regulates in a different manner normal and Crouzon osteoblast phenotype. Moreover, FGF2 could act through an autocrine cascade that involves an altered production of ILs. This lead to the possibility that FGF2 and ILs are also *in vivo*

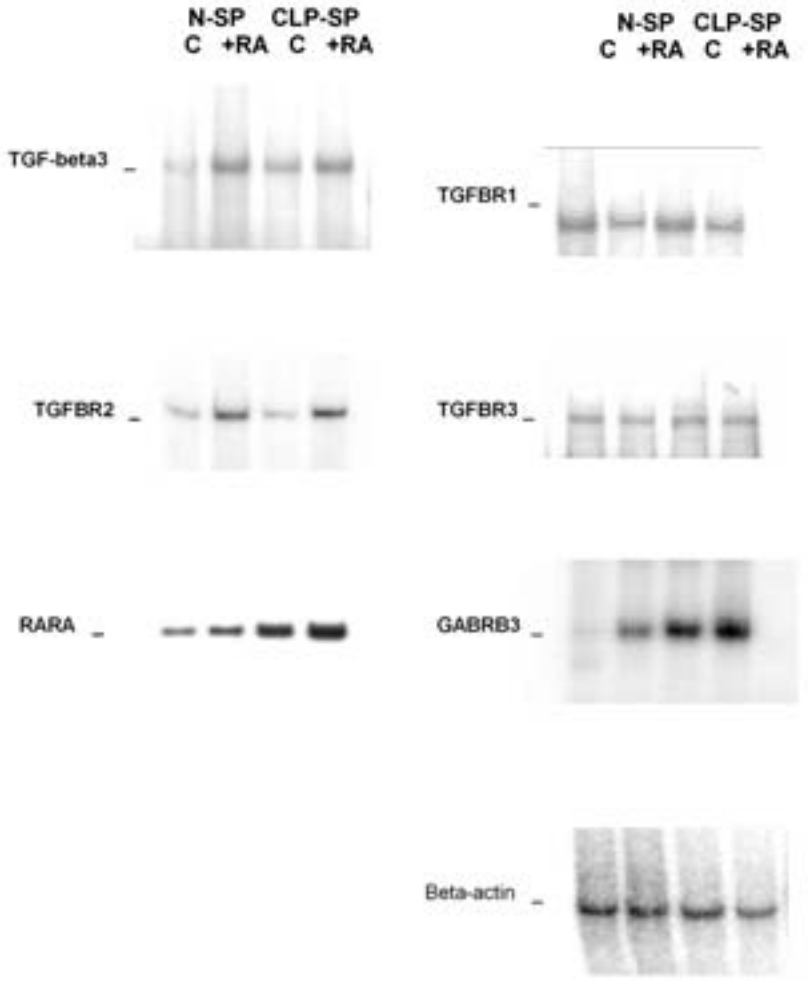

Figure 2. Representative samples of the semiquantitative radioactive RT-PCR used to quantitate the mRNA levels of different specific genes. Beta-actin was used as internal control in all PCRs. C and RA represent untreated and 10μM RA-treated fibroblasts respectively. The amplification products were electrophoresed on 6% polyacrylamide gels. Gels were dried and exposed for electronic autoradiography. Values of semiquantitative analysis are reported in Table 3. Similar results were seen in four independent experiments for each of the 4 patients; each experiment was performed in quadruplicate. Figure (from Baroni, 2006) reproduced with the kind permission of The Feinstein Institute for Medical Research.

jointly involved in the bone-remodelling microenvironment as local coupling factors. In another work (Bodo, 2000), we provided the first evidence that fibroblasts obtained from patients affected by Crouzon syndrome retain their capacity to respond to FGF2, despite mutations in the high-affinity FGF2 receptor. The growth factor reduces IL-1 secretion, down-regulates biglycan and procollagen alpha, and increases betaglycan gene expressions. Since betaglycan is a co-receptor for FGF2 signaling, we suggested an alternative signal transduction pathway in Crouzon fibroblasts to explain the documented changes in ECM macromolecule production. Finally, we analyzed the role of some FGF signalling molecules involved in FGFR2 regulation and their effects on the ECM (Bodo *et al.,* 2002). Compared with normal fibroblasts, excess fibronectin catabolism is present in Crouzon fibroblasts and differences were more marked when FGF2 was added. Very few phosphoproteins were visible in anti-Grb2 immunoprecipitations from Crouzon fibroblasts, which showed a significant increase in the number of high affinity and low-affinity FGF2 receptors. These results suggest that the abnormal genotype and the Crouzon cellular phenotype are related. To compensate the low levels of tyrosine phosphorylation, Crouzon cells might increase the numbers of FGFR2, thus increasing the cell surface binding sites for FGF2.

Non-syndromic CLP

We studied (Bodo *et al.,* 1999b) TGF-α, TGF-β, and TGF-β3 expressions and their effects on ECM macromolecule production of normal and cleft palatal fibroblasts *in vitro,* to investigate the mechanisms by which the phenotypic modulation of fibroblasts occurs during the cleft palate process. TGF-β isoforms and ECM components were dif-

Table 3. Semiquantitative analysis of mRNA for TGF-beta3, TGFBR1, TGFBR2, TGFBR3, RARA and GABRB3 in normal and CLP fibroblasts treated or not with RA.

	Normal fibroblasts		*CLP fibroblasts*	
	Control	*RA*	*Control*	*RA*
TGF-β3	100±13	242±27*	129±15†	205±23*
TGFBR1	100±13	65±7*	93±10‡	51±6*
TGFBR2	100±11	267±28*	100±10‡	257±27*
TGFBR3	100±12	95±11 NS	96±12‡	111±13 NS
RARA	100±14	170±19*	284±32§	459±49*
GABRB3	100±11	307±35*	486± 53§	531±61 NS
Actin	100±11	95±10 NS	90±11‡	82±10 NS

*The values indicate mRNA levels, corrected for beta-actin mRNA levels and expressed as the percentage of untreated normal fibroblasts. All values are mean ± SD of four separate experiments performed in quadruplicate. The results are analysed by ANOVA. Differences of CLP fibroblasts vs. normal fibroblasts: §F-test significant at 99%; †F-test significant at 95%; ‡not significant. Differences vs. control: *F-test significant at 99%; NS, not significant. Table (from Baroni), 2006 reproduced with the kind permission of The Feinstein Institute for Medical Research.*

ferently expressed and were correlated to the CLP phenotype. In particular, CLP fibroblasts produced more GAG and collagen than normal fibroblasts and when all three TGF-β isoforms were added, ECM production increased even more. Thus, strength was given to the hypothesis that TGF-β isoforms are the potential inducers of phenotypic expression in palatal fibroblasts during development and that an autocrine growth factor production mechanism may be responsible for the phenotypic modifications. TGF-β is also involved in regulating the interleukin network and IL-1 and IL-6 in particular (Bodo *et al.,* 1998b; Schluns *et al.,* 1997). IL-6 is a multifunctional cytokine, which, unlike TGF-β, reduces connective macromolecule production (Roodman *et al.* 1992). Interactions between IL-6 and TGF-β3 trigger a cascade of events that control developmental processes. We speculated that a concerted action of TGF-β3 and IL-6 promotes the ECM composition of the CLP fibroblast phenotype. To test this hypothesis, we examined collagen, GAG and biglycan proteoglycan (PG) synthesis in response to IL-6 and determined how IL-6 production and biglycan expression were modified in CLP fibroblasts after TFG-β3 exposure. Our data (Baroni, et al. 2002, 2003) suggested the increase in matrix components that characterize the CLP fibroblast phenotype might be due to a concerted TGF-β3-IL-6 action. We hypothesized changes in cross-talk between TGF-β3 and IL-6 signal transduction pathways are involved in the induction of cleft palate.

During embryogenesis, retinoic acid (RA) and gamma-aminobutyric acid (GABA)ergic signaling systems are also potentially involved. We aimed to verify the presence of phenotypic differences between primary cultures of secondary palate (SP) fibroblasts from 2-year old subjects with familial non-syndromic cleft lip and/or palate (CLP-SP fibroblasts) and age-matched normal SP (N-SP) fibroblasts (Baroni *et al.,* 2006). The effects of RA which, at pharmacologic doses, induces cleft palate in newborns of many species were also studied. We demonstrated for the first time that GABA receptor (GABRB3) mRNA expression was upregulated in human CLP-SP fibroblasts (Figure 2) (Table3). RA treatment increased TGF3 and RARA gene expression in both cell populations but upregulated GABRB3 mRNA expression only in N-SP cells (Figure 2) (Table3). These results show that CLP-SP fibroblasts exhibit an abnormal phenotype *in vitro*, respond differently to RA treatment and suggest that altered cross-talk between RA, GABAergic and TGF-β signaling systems could be involved in human cleft palate fibroblast phenotype. Hence, normal orofacial configuration is the end-product of highly regulated interplay between ECM molecules and cells from the epithelium and mesenchyme which produce growth factors such as the TGF-β family members (TGF-β1, TGF-β2, and TGF-β3). All three mammalian TGF-β isoforms are expressed during palatal development and exact timing and spatial expression are required. TGF-β3 appears to play a pivotal role, since TGF-β3 gene mutations and/or deficiences give rise to cleft palate in humans (Lidral *et al.,* 1998) and mice (Kaartinen *et al.,* 1995). Our data extend previous findings (Bodo *et al.,* 1999b; Baroni *et al.,* 2003) that CLP-SP fibroblasts retain an abnormal phenotype *in vitro* which we have studied in terms of ECM production, TGF-β system, RARA and GABRB3 expression and different response to RA. The results contribute to a better understanding of the interactions between RA and TGF-β signaling pathways and support the hypothesis that altered cross-talk between TGF-β and RA signaling systems plays a role in eliciting the CLP phenotype in humans.

Our group has investigated the possible involvement of genes coding for growth factors in the etiology of CLP in recent years. The earlier investigations regarded TGFA gene, which was studied by both allelic association and linkage analyses (Scapoli et al, 1998; Pezzetti *et al.*, 1998). We

observed no allelic association with the Taq I polymorphism, however genetic linkage between microsatellite markers and putative disease locus was detected in a subset of families. Taking together these data suggest a possible role of TGFA gene or a nearby gene in CLP onset.

On investigating the TGFB3 locus (14q24), our group obtained only borderline results, thus we were unable to distinguish whether this gene contributed or not to the etiology of CLP in our sample (Scapoli 2002). On the other hand, our family based investigation, even if with slight statistical evidence, supports a role for the RARA gene in CLP disease (Scapoli 2002). Interestingly, our group observed a significant relationship between the β 3 subunit of the gamma-aminobutyric acid receptor (GABRB3) and CLP, suggesting that the GABRB3 gene is involved in this congenital disease. Although GABR is the target of benzodiazepine, none of our patients presented neurologic diseases. In the same study, it was also demonstrated that the GAD1 gene, which encodes the GABA-producing enzyme, is not involved in CLP pathogenesis.

Conclusions

Taken together, these data suggest that the changes in the distribution of ECM components participate in the regulation of the complex morphogenetic events that occur during cranial and orofacial development. Several growth factors are involved in this cascade of events, each playing a role in the commitment of calvaria and orofacial cells to different phenotypes. The balance among ECM components, cytokines and growth factors as FGF2 and TGF-β probably determines the degree and extent of induced cellular response. Research into the mechanisms regulating this balance has entered an exciting phase also thanks to cultures from Apert, Crouzon and CLP patients that provide a promising model for these studies in view of therapeutic strategies as a complement to surgery.

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The nuclear envelope, human genetic diseases and ageing

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Here we present an overview of the experimental evidence and of the conceptual basis for the involvement of lamins and nuclear envelope proteins in a group of genetic diseases collectively referred to as laminopathies. Some of these diseases affect a specific tissue (skeletal and/or cardiac muscles, subcutaneous fat, peripheral nerves), while others affect a variety of tissues; this suggests that the pathogenic mechanism of laminopathies could reside in the alteration of basic mechanisms affecting gene expression. On the other hand, a common feature of cells from laminopathic patients is represented by nuclear shape alterations and heterochromatin rearrangements. The definition of the role of lamins in the fine regulation of heterochromatin organization may help understanding not only the pathogenic mechanism of laminopathies but also the molecular basis of cell differentiation and ageng.

Key words: nuclear envelope, heterochromatin, laminopathies, prelamin A, ageing.

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In the last twenty years our research group has been interested in the study of the molecular organization of the cell nucleus. These investigations have been performed by using a combined approach of biochemical, cytochemical, and ultrastructural procedures in order to obtain a comprehensive design of the morphology and function of the different intranuclear compartments. The cell nucleus presents an organization at least complex as the cytoplasm; furthermore, whilst the cytoplasm can be subdivided into cell membrane delimited compartments, intranuclear structures are not membrane-bounded and are frequently intermixed. These structures can be defined *nuclear domains* when can be identified by light and/or electron microscopy, visualized *in vivo* by GFP-tagged constructs, isolated in an enriched form to be biochemically analyzed, and characterized by a specific class of stably associated proteins. Once identified, the nuclear domains can be studied in a dynamic way, in order to determine their functions.

Our research group contributed to the knowledge of some aspects of the functional organization of the main recognized nuclear domains, including the nuclear matrix (Maraldi *et al.*, 1986; Tait *et al.,* 1998), the nucleolus (Zini *et al.*, 1994), the splicing domain (Maraldi *et al.,* 1999a; Bavelloni *et al.*, 2006), the chromosome territories (Cinti *et al.*, 1993; Squarzoni *et al.*, 1994; Maraldi *et al.*, 1999b), and the nuclear signal transduction system (Maraldi *et al.*, 1992; Mazzotti *et al.*, 1995; Maraldi *et al.*, 1999a; Maraldi *et al.*, 2000).

In this report, we present the main advance we obtained in the study of the nuclear envelope and, in particular, in its involvement in the pathogenesis of a variety of human genetic diseases.

The nuclear envelope

The cell nucleus is delimited by the nuclear envelope (NE), constituted by the outer nuclear membrane (ONM), which is part of the endoplasmic

reticulum, by the inner nuclear membrane (INM), devoid of ribosomes and presenting a set of specific proteins, and by the nuclear lamina (NL). Our studies have been particularly devoted to the functions of the INM-associated proteins and of the lamins, that are expressed into the nuclear lamina.

The NL appears as a continuous structure, with a thickness variable from 10 to 300 nm, located between the INM and the peripheral heterochromatin. The ultrastructural analysis identified a 3D organization of the nuclear lamina *in situ* only in the *Xenopous* oocytes, where it appears as a net with square meshes formed by 10 nm thick filaments (Burke and Stewart, 2002). We demonstrated a similar organization in rat liver isolated nuclei, analyzed by freeze-fracturing (Maraldi *et al.,* 1986).

The NL is constituted by type V intermediate filaments, the lamins; type B lamins are expressed in almost all the cells, whilst A type lamins are expressed in a tissue specific way during cell differentiation. Nuclear lamins undergo transition from the polymerized to the un-polymerized state, thus contributing to the NE breaking and formation at each cell cycle. The first step of the latter process requires lamin B1 interaction with condensed chromosomes in telophase, the following recruitment of membrane vesicles capable of interacting with the nuclear pore complexes (NPCs) and to fuse to form the perinuclear cisterna, and then the contribute of lamins A/C to assemble the nuclear lamina. The assembly of type B lamins with lamins A/C is essential for the correct NPCs organization, through the interaction of lamin B1 and the NPC-associated protein NUP153 (Holaska *et al.,* 2002).

The NE formation also depends on the presence of a wide set of INM-associated proteins and of some chromatin-associated proteins. The INM-associated proteins, once synthesized in the RER, have to interact with lamins or with chromatin, or both, to be integrated into the INM (Zastrow *et al.,* 2004). Among the INM-associated proteins, those containing the LEM domain, that is LAP2, emerin, and MAN1, interact with the lamins as well as with some chromatin-associated proteins, including BAF and HP1. The INM-associated protein LBR, which lacks the LEM domain, also interacts with both lamin B and HP1, as well as with DNA and H3/H4 histones. LAP2α is mainly located in intranuclear regions and interacts with lamin A/C and chromatin, whilst the other isoforms of the protein are exclusively present at the nuclear lamina level. The INM-associated proteins participate to the NE assembly and to the chromosome decondensation, being initially LAP2β and LBR involved into an interaction with lamins in non-centromeric regions of the chromosomes, and then LAP2α and emerin in the centromeric regions (Shumaker *et al.,* 2003; Gruenbaum *et al.,* 2005).

Since lamin immunodepletion or the expression of dominant negative lamins induce the block of replication, lamins are conceivably interacting with replication complexes (Zastrow *et al.,* 2004). Furthermore, the strict association of the nuclear lamina with the heterochromatin suggests that lamins could contribute to the repression of gene transcription. Gene-rich chromosome domains, indeed, are generally located in inner zones of the nucleus, whilst gene-poor regions are located close to the nuclear lamina. On the other hand, both transcription and RNA processing require a correct expression and an intranuclear localization of lamins, suggesting that these nuclear activities require the presence of a nucleoskeletal structure containing lamins (Gruenbaum *et al.,* 2005).

Interestingly, some transcription factors have been localized at the nuclear lamina, where they interact with lamins or INM-associated proteins. Most of these transcription factors are inhibitory, such as Oct-1, pRb, GCL, and SREBP1 (Maraldi and Lattanzi, 2005). Finally, the documented interactions of lamins with proteins that are involved in the chromatin remodelling, such as HP1, H3/H4 histone tetramers, and the nuclear actin bound to the SWI/SNF remodelling complex, suggest that transcription could be repressed by affecting the whole conformational arrangement of the chromosome domains (Maraldi *et al.,* 2004).

The induction phases of apoptosis require lamin proteolysis, which is preceded by lamin phosphorylation through PKCα and PKCδ. Lamin hydrolysis by caspases precedes DNA fragmentation and the lysis of INM-associated proteins, and the cells in which the lamin expression has been reduced undergo apoptotic alterations (Holaska *et al.,* 2002).

It is evident, therefore, that lamins and INM-associated proteins, not only contribute to the NE assembly, but play a variety of functions, which are essential for the control of cell viability, replication and differentiation. As a consequence, the altered expression of these nuclear envelope proteins could result in diseases. In recent years, a wide range of inherited diseases, collectively termed *nuclear envelopathies* if mutations arise in INM-associated

proteins, or *laminopathies* if mutations arise in lamins, have been identified. Therefore, attention has been focused on the molecular characteristics of these NE components, in order to clarify the pathogenic mechanisms that could account for the complexity of the observed phenotypic alterations found in these diseases (Maraldi *et al.,* 2002).

Nuclear envelopathies and laminopathies present an impressive variety of disease phenotypes but common nuclear alterations

At the moment, disease-causing mutations have been reported for seven genes coding for nuclear envelope proteins, i.e. *EMD, LMNA, ZMPSTE24, LBR, MAN1, LAP2*, and *AAAS* (Broers *et al.,* 2004). Our interest has been mainly devoted to one nuclear envelopathy, i.e. the X-linked form of Emery-Dreifuss muscular dystrophy XL-EDMD, due to mutation of the *EMD* gene, coding for emerin (Maraldi *et al.,* 2002; Maraldi and Merlini, 2004), and to the large group of primary laminopathies, due to mutation of the *LMNA* gene, coding for lamin A/C (Maraldi *et al.,* 2004). Primary laminopathies include at least ten different diseases in which specific tissues are affected in isolated fashion, or several tissues are systemically involved; according to these criteria laminopathies can be grouped into five classes: 1) striated muscle; 2) peripheral nerves; 3) adipose tissue; 4) premature ageing; 5) overlapping syndromes (Broers *et al.,* 2006).

The striated muscle laminopathies include AD-EDMD and AR-EDMD, the autosomal and recessive forms of the Emery-Dreifuss muscular dystrophy, which present skeletal and cardiac muscle involvement as well as joint contractures, almost identical to those found in XL-EDMD. Other striated muscle laminopathies are the dilated cardiomyopathy with conduction system defects (DCM-CD), characterized by progressive conduction system disease and dilated cardiomyopathy without skeletal muscle involvement, and the limb-girdle muscular dystrophy type 1B (LGMD1B), with cardiological abnormalities and proximal muscle weakness and wasting.

The laminopathies with peripheral nerve involvement include the Charcot-Marie-Tooth type 2 (CMT2B1), characterized by the demyelinization of motor nerves with a secondary wasting of the distal lower limb muscles and the autosomal dominant axonal Charcot-Marie-Tooth disease (AD-CMT2), characterized by an axonal involvement associated with muscular dystrophy, cardiac disease and partial lipodystrophy.

Partial lipodystrophy due to mutation of the *LMNA* gene is an autosomal dominant disorder referred to as familial partial lipodystrophy of Dunningan type (FPLD). Patients, with the onset of puberty, present an almost complete loss of subcutaneous fat from the upper and lower extremities, and gluteal and truncal areas, whilst fat accumulates on face and neck, as well as in the intra-abdominal regions. Patients are insulin resistant and may develop diabetes, hypertriglycideridemia and atherosclerotic vascular diseases. A further disease, identified as type A insulin resistance syndrome, characterized by polycystic ovary with severe hyperandrogenism, and severe insulin resistance, is also due to mutation of the LMNA gene, restricted at a single aminoacid (G602S). Interestingly, mutations occurring in FPLD affect residues at the surface of the Ig fold domain downstream of the nuclear localization signal (NLS) of the lamin A, whilst those found in striated muscle and peripheral nerve laminopathies are mainly located in the central rod domain upstream of the NLS (Hegele, 2005).

The very intriguing class of systemic laminopathies has been recently identified, which include the mandibuloacral dysplasia (MAD-A), the Hutchinson-Gilford progeria syndrome (HGPS), the atypical Werner syndrome (WRN-like), the generalized lipoatrophy, insulin-resistant diabetes, leukomelanodermic papules, liver steatosis, and hypertrophic cardiomyopathy (LIRLLC), the restrictive dermopathy (RD), and the lethal fetal akinesia (SFAk). In MAD-A, lipodystrophy is associated to skeletal abnormalities, such as mandibular and clavicular acroosteolysis, joint contractures, delayed closure of cranial suture, and postnatal growth retardation. HGPS and WRN-like are premature ageing syndromes, in which, besides the symptoms present in MAD-A, other tissues and mainly the vascular system undergo a premature ageing. HGPS patients, as a consequence of widespread atherosclerosis, die at a median age of 13 years. On the other hand, RD and SFAk are characterized by an impressive variety of systemic disorders so that they are lethal before or within few days after birth.

Finally, there are an increasing number of cases in which *LMNA* mutations have been reported to occur in individuals or families harbouring several tissue involvements, suggesting the presence of an overlapping continuum within the different types of laminopathies.

The impressive amount of disease phenotypes of laminopathies rises the question of how mutations in a gene, which is expressed in nearly every differentiated cell and codes for a structural protein of the nucleus, could give rise to such a variety of tissue-restricted pathologies.

Laminopathy disease models

Lamins provide a structural scaffold that maintains the nuclear integrity, but they are also interacting with nuclear proteins that modulate chromatin arrangement, gene expression and cell cycle progression. On the basis of these considerations, different models, that could account for a pathogenic mechanism of laminopathies, were proposed.

The first model suggests that mutant lamin A results in alteration of the nuclear lamina mechanical properties, and mainly of its resistance to mechanical stress (Hutchison *et al.,* 2001). This could lead, in particular in contractile tissues, to nuclear damages and cell death, provided that abnormal lamin assembly may destabilize the interactions between the nuclear and the cytoskeletal networks (Broers *et al.,* 2004).

Another model proposes that mutant lamin A causes misregulation of different tissue-specific gene expression, either directly or at the epigenetic level. In fact several transcriptional regulators have been found to interact with lamins and their influence on cell cycle progression and differentiation may be affected by the expression of mutant lamins (Zastrow *et al.,* 2004). The details of the molecular mechanisms underlying this regulation remain unclear, but lamin-dependent regulation of gene expression could occur at the epigenetic level, by modulating heterochromatin organization (Maraldi *et al.,* 2005).

A further model proposes that mutations in *LMNA* impair the balance between cell proliferation and differentiation that represents the regulatory mechanism controlling adult stem cells involved in tissue regeneration (Gotzmann and Foisner, 2005).

The proposed models are not mutually exclusive, and it is possible that a combination of these mechanisms may contribute to various degrees to the diverse disease phenotypes observed in laminopathic patients. It was initially thought that mutated or incorrectly spliced lamin variants result in a loss-of-function phenotype; as a consequence, the structural model, in which a loss of lamin stability could account for nuclear fragility, was consistent with the observed muscle tissue alterations. However, since almost all the inherited changes in *LMNA* are heterozygous, it is assumed that the mutated protein dominantly affects the structure and/or the function of the wild-type lamin A expressed by the unaffected allele, resulting in a gain-of-function phenotype.

In support of this type of pathogenic model, recently we have obtained experimental evidence in FPLD (Capanni *et al.,* 2005). It has been demonstrated that in HGPS (De Sandre-Giovannoli *et al.,* 2003) and RD (Navarro *et al.,* 2004), mutations in the *LMNA* gene activate a cryptic splice site leading to the expression of a shortened, incompletely processed prelamin A that accumulates in the nucleus (Goldman *et al.,* 2004). We demonstrated that also in FPLD there is a nuclear accumulation of incompletely processed prelamin A and that this mutant lamin A form is able to sequester the transcription factor sterol response element binding protein 1 (SREBP1), required for adipogenesis and thus negatively affecting adipocyte differentiation as occurs in FPLD patients and in other laminopathies in which lipodystropic changes also occur (Capanni *et al.,* 2005). Furthermore, the introduction of wild-type lamin A in cells derived from HGPS patients was unable to rescue the abnormal phenotype, indicating that the accumulation of mutant not correctly processed lamin A variants, instead of a partial loss of the wild-type protein, result into toxic effects that impair key nuclear functions.

However, since prelamin A accumulation does not occur in all laminopathies, is there possible to find a detectable marker that allows one to determine whether laminopathies present a common pathogenic mechanism?

Heterochromatin altered pattern in laminopathies

Our group contributed to the characterization of the phenotypic alterations of the firstly discovered nuclear envelopathy, that is XL-EDMD, due to mutation of the INM-associated protein emerin (Bione *et al.,* 1994). In fact, we provided evidence that emerin is connected with the nuclear matrix (Squarzoni *et al.,* 1998) and could account for some nuclear and chromatin alterations consisting in changes in the nuclear profile with deep indentations and in focal loss and detachments of the peripheral heterochromatin from the nuclear lamina (Ognibene *et al.,* 1999). Therefore, we focused attention on the nuclear envelope related mechanisms that modulate the chromatin arrangement through chromatin

remodelling complexes (CRCs), responding to nuclear inositide signals (Maraldi *et al.,* 2002).

In higher eukaryotes, indeed, CRCs are multi-subunit protein complexes involved in the control of gene expression. Among them, BAF complex (Brahma-related gene associated factors), is constituted by BRG1, β-actin, and the actin-related protein BAF53; its activation, leading to chromatin decondensation, is triggered by intranuclear increase of PI(4,5)P2 levels, that modulate actin polymerization, by displacing BAF53 (Zhao *et al.,* 1998). It has been suggested that mutations in lamin A/C or emerin can affect gene expression through proteins having dynamic properties, such as nuclear actin, resulting in modulating the chromatin arrangement (Maraldi *et al.,* 2002). In particular, the failure to correctly confine transcriptionally inert chromatin at the nuclear periphery may affect gene expression in crucial moments of cell differentiation, resulting in defect in tissue regeneration in the adult organism (Maraldi *et al.,* 2004).

The possibility that lamins and actin interact inside the nucleus has been experimentally demonstrated; moreover it has been found that this interaction is regulated by phosphorylation along myoblast differentiation (Lattanzi *et al.,* 2003), suggesting that nuclear actin is a biologically relevant partner for emerin and lamin A during myogenesis. Therefore, it is conceivable that actin oligomers constitute architectural partners for lamins, influencing chromatin arrangements, and directly or indirectly, gene regulation (Zastrow *et al.,* 2004). Subtle alterations in chromatin arrangement affecting gene expression, however, might not necessarily affect all cell types, but mainly long-lasting cells which present long quiescent periods with sudden activation phases; such changes may require deep chromatin remodelling and the reprogramming of the whole nuclear size and shape, as occurs in most of the cells affected in laminopathies, including muscle cells, neurons and adipocytes (Maraldi *et al.,* 2004).

Heterochromatin patterns characterize distinct classes of laminopathies

Altered pattern of heterochromatin distribution has been, so far, identified in several laminopathies, including EDMD2 (Sabatelli *et al.,* 2001), LGMD1B, FPLD (Capanni *et al.,* 2005), MAD (Filesi *et al.,* 2005) and HGPS (Columbaro *et al.,* 2005). It is conceivable that mutations affecting lamin A gene result in defective interactions of the nuclear envelope with chromatin-associated proteins, such as HP1, thus impairing the correct localization of heterochromatin at the nuclear periphery. This, in turn, might affect the silencing of genome regions required to perform a differentiation-related program of gene repression. In fact, in MAD cell nuclei, for example, we found that HP1β and three-methylated histone H3 (H3K9) became partially soluble by Triton X-100 treatment, and a redistribution of LBR, a nuclear envelope protein interacting with HP1, suggesting that heterochromatin was partly unstructured, as indicated by ultrastructural analysis (Filesi *et al.,* 2005). In fact, a typical feature of MAD as well as HGPS nuclei, when compared to other laminopathies, is the almost complete absence of the heterochromatin. Therefore, in these cases, mutations affecting lamin A appear to interfere with the correct assembly and/or stability of the heterochromatin-associated complex constituted by H3K9, HP1β and LBR (Columbaro *et al.,* 2005). Also in FPLD, abnormally decondensed chromatin areas are present close to the nuclear lamina, as well detachments of the chromatin from the lamina (Capanni *et al.,* 2003). In all these cases, the nuclear defects appear to be not related to a loss of mature wild-type lamin A, which is only slightly reduced. Furthermore, mutations are mainly localized at the lamin A/C C-terminus, mainly interacting with non-nucleoskeleton elements, such as DNA, chromatin-associated proteins and transcription factors (Hegele, 2005).

On the other hand, in laminopathies affecting muscle such as EDMD1,EDMD2, CMD1A and LGMD1B, defective lamin phosphorylation (Cenni *et al.,* 2005), nuclear envelope profile defects and focal loss or detachment of peripheral heterochromatin (Ognibene *et al.,* 1999; Sabatelli *et al.,* 2001; Maraldi *et al.,* 2005) are common features independent of the site at which mutations occur. In these cases, a loss of mature wild-type lamin A or emerin (in EDMD1) appears to be involved in the nuclear instability; furthermore, mutations are mainly detectable in the central rod domain of the lamin A, involved in the stability of the assembled nucleoskeletal elements (Hegele, 2005).

These experimental findings, based on the phenotypical appearance of nuclear defects, which appear in some way related to the mutation position within the *LMNA* sequence, appear to predict different pathogenic mechanisms and/or organ system

involvement in at least two distinct classes of laminopathies.

Heterochromatin and ageing: a lesson from laminopathies

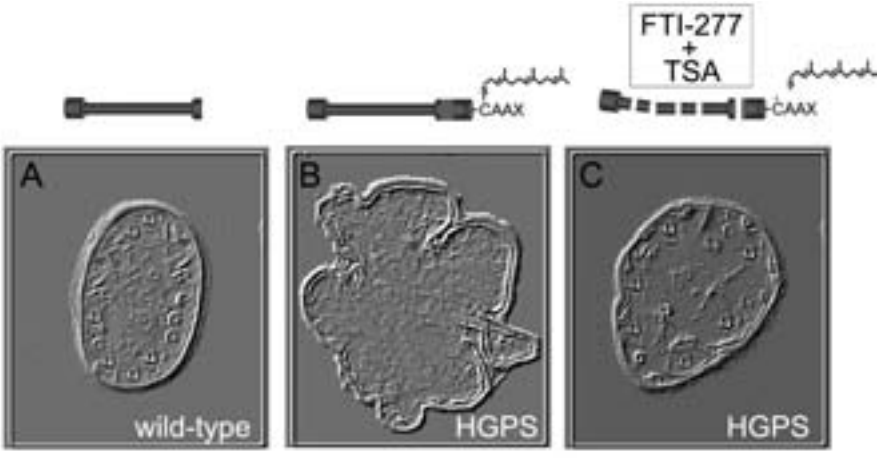
The most striking feature of non-muscular laminopathies is the fact that nuclear and chromatin defects appear not to be due to a loss of mature lamin A; in fact the over-expression o wild-type lamin A did not rescue nuclear alterations (Scaffidi and Misteli, 2005). This suggests that nuclear defects, and the heterochromatin loss are not due to a loss a functional lamin A, but conceivably to a dominant negative effect of accumulating un-properly processed lamin A. This possibility has been largely confirmed by experimental data accumulated in the last two years.

The first hint to this pathogenic model was provided by the finding that also heterozygous single point mutations in LMNA linked to FPLD induce a progressive accumulation of incompletely processed prelamin A (Capanni *et al.,* 2005), suggesting that at least a subset of laminopathies might be caused through aberrant accumulation of prelamin A. Therefore, a dominant negative effect of mutated prelamin A seems to account for the observed disease phenotype. In support of a gain-of-function, instead of a loss-of-function phenotype, the prelamin A accumulation was found to result, in FPLD cells, in a binding of the transcription factor sterol response element binding protein 1 (SREBP1). The recruitment by prelamin A of SREBP1 that is required for adipogenesis could negatively affect adipocyte differentiation (Capanni *et al.,* 2005; Maraldi *et al.,* 2006).

A further hint for the understanding of prelamin A role in the modulation of heterochromatin arrangement, was obtained by analyzing the effect of farnesyl transferase inhibitors which impair subsequent processing of lamin A precursor protein by endoproteases such as the metalloprotease ZMPSTE24, which , when mutated, gives rise to MAD (Agarwal *et al.,* 2003). In fact, we obtained evidence that prelamin A accumulation by farnesyl transferase inhibitors in myoblasts caused nuclear lamina invagination and chromatin arrangement reorganization (Maraldi *et al.,* 2004). Moreover, accumulation of incompletely processed prelamin A has been demonstrated to occur in FPLD (Capanni *et al.,* 2005) and MAD (Filesi *et al.,* 2005). In this case, accumulation of prelamin A resulted into an altered distribution of LBR and in the destabilization of HP1β and of H3K9. These changes can account for a complete heterochromatin remodelling that could represent a key event in the epigenetic changes involved in the pathogenesis of systemic laminopathies (Filesi *et al.,* 2005).

This pathogenic model was further confirmed also in other laminopaties, including those characterized by premature senescence phenotype. The two premature ageing diseases are HGPS (progeria of childhood) an Werner's syndrome (progeria of adults). Most cases of HGPS result from a Gly608Gly mutation that forms an ectopic mRNA splicing site leading to the expression of a truncated prelamin A lacking 50 amino acids within its tail domain. This mutant protein termed LAΔ50 or progerin (Goldman *et al.,* 2004) lacks the second proteolytic cleavage site for the processing of lamina and the mature protein contains eight residues of prelamin A and is farnesylated. Fibroblasts from HGPS patients, when propagated in culture, undergo typical changing in nuclear shape, including lobulation of the nuclear envelope, thickening of the nuclear lamina, clustering of the nuclear pore complexes and almost total loss of peripheral heterochromatin (Columbaro *et al.,* 2005). Thus, we proposed that a key element of chromatin-remodeling complexes may be prelamin A, whose post-translational modifications may serve as regulatory mechanisms affecting higher order chromatin organization (Maraldi and Lattanzi, 2005).

A direct demonstration that nuclear alterations in HGPS are caused by a concentration-dependent dominant-negative effect of unprocessed prelamin A has been obtained by the expression of the mutant lamina in normal cells, which results in similar nuclear alterations (Goldman *et al.,* 2004). On the contrary, silencing of the mutant mRNA has been demonstrated to down-regulate prelamin A expression and to reverte the nuclear phenotype (Scaffidi and Misteli, 2005). The dominant-negative effect of prelamin A expression may be attributed to the farnesyl moiety retained at the C terminus as a result of the second proteolytic cleavage site involved in the processing being missing (Glynn and Glover, 2005). We obtained a strong experimental evidence supporting this pathogenic model by treating HGPS fibroblasts with the farnesyl transferase inhibitor mevinolin in combination with the histone deacetylase inhibitor Trichostatin A. In fact, by this farmacological treatment, the progeric altered nuclear



Nuclear morphological abnormalities
Disorganization of heterochromatin

Figure 1. The dominant-negative effect of farnesylated prelamin A in cells from HGPS patients is recovered by a pharmacological treatment. The drawing depicts: A) Mature lamin A is expressed in wild-type cells (top), whose nuclei exhibit a continuous profile and patches of peripheral heterochromatin; B) Progerin, a farnesylated prelamin A form (top), accumulates in HGPS cell nuclei which result enlarged, with blebs and indentations, and almost devoid of heterochromatin; C) The treatment with FTI-277 and TSA results in a dramatic reduction of progerin (which, as indicated, is possibly de-farnesylated and fragmented); both the nuclear profile and the heterochromatin pattern appear recovered by the pharmacological treatment.

phenotype was completely reverted to a normal one, and the reduced transcriptional rate of HGPS nuclei was reported to normal levels (Columbaro *et al.,* 2005).

These results not only confirm that, in a large group of laminopathies, including systemic progeric syndromes, chromatin alterations are due to a dominant-negative effect of accumulating mutant unprocessed prelamin A, but that the main effects on nuclear arrangement and on transcriptional activity can be rescued by a pharmacological treatment that is able to reduce the stability of prelamin A by interfering with its farnesylation (Figure 1). Since some farnesyl transferase inhibitors are already in phase II and III clinical trials and appear to be well tolerated (Young *et al.,* 2005), a possible drug treatment may be useful in the treatment of HGPS patients, and, possibly in other laminopathies.

Progeric laminopathies appear to represent an accelerated model of normal ageing, being almost all tissues involved into progressive degenerative processes. This could be due to a reduction of a definite life span of cells committed to differentiation programs before they enter senescence. Age-dependent alterations can be observed to occur at a phenotypic level in the nuclear organization also in very primitive organisms. In fact, in C. *elegans*, the nuclear architecture undergoes in most non-neuronal cell types progressive age-dependent alterations, including lobulation of the nuclear envelope and heterochromatin rearrangement (Haithcock *et al.,* 2005). Since these changes resemble those occurring in HGPS fibroblasts, and appear to involve changes in lamin A processing, this could represent a physiological mechanism to regulate cell senescence (Mattout *et al.,* 2006).

In conclusion, mutations of a gene coding for a simply *structural* nuclear envelope protein such as lamin A/C result in an astonishing variety of functional, systemic diseases disclosing the possibility that impairment of post-translational processes of this protein may be at the basis not only of this group of devastating diseases but also of physiological ageing mechanisms. As a consequence, an increasing interest is expected to develop in the field of nuclear lamins structure and function especially in regulation of transcription, chromatin remodelling, and ageing.

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Nuclear phosphatidylinositol 3,4,5-trisphosphate, phosphatidylinositol 3-kinase, Akt, and PTEN: emerging key regulators of anti-apoptotic signaling and carcinogenesis

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Inositol lipid-derived second messengers have long been known to have an important regulatory role in cell physiology. Phosphatidylinositol 3-kinase (PI3K) synthesizes the second messenger 3,4,5'-phosphatidylinositol trisphosphate (PtdIns 3,4,5P3) which controls a multitude of cell functions. Down-stream of PI3K/PtdIns 3,4,5P_3 is the serine/threonine protein kinase Akt (protein kinase B, PKB). Since the PI3K/PtdIns 3,4,5P_3 /Akt pathway stimulates cell proliferation and suppresses apoptosis, it has been implicated in carcinogenesis. The lipid phosphatase PTEN is a negative regulator of this signaling network. Until recently, it was thought that this signal transduction cascade would promote its anti-apoptotic effects when activated in the cytoplasm. Several lines of evidence gathered over the past 20 years, have highlighted the existence of an autonomous nuclear inositol lipid cycle, strongly suggesting that lipids are important components of signaling pathways operating at the nuclear level. PI3K, PtdIns(3,4,5)P_3, Akt, and PTEN have been identified within the nucleus and recent findings suggest that they are involved in cell survival also by operating in this organelle, through a block of caspase-activated DNase and inhibition of chromatin condensation. Here, we shall summarize the most updated and intriguing findings about nuclear PI3K/PtdIns(3,4,5)P_3/Akt/PTEN in relationship with carcinogenesis and suppression of apoptosis.

Key words: PtdIns(3,4,5)P_3; PI3K; Akt; nucleus; apoptosis; cancer; PTEN.

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Transferring of signals from the plasma membrane to the cell nucleus is an extremely complex multistep process which strongly depends, among other molecules, on PtdIns lipid signaling molecules (Di Paolo and De Camilli, 2006). The repertoire of cellular processes known to be directly or indirectly regulated by this class of lipids has now dramatically expanded. Inositol phospholipids are concentrated at the cytosolic surface of membranes where they are substrates for phospholipases, kinases, and phosphatases. Among lipid kinases, PI3K has emerged as a key regulator of multiple signaling cascades, being involved in the control of many critical cell responses (Engelman *et al.,* 2006). PI3K synthesizes four species of non-canonical, 3'-phosphorylated inositides: PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns (3,4,5)P_3. Several lines of evidence indicate that members of PI3K family can also be considered as oncogenes, because they control cell cycle progression, differentiation, survival, invasion and metastasis, and angiogenesis (Cully *et al.,* 2006). Many biological effects of PI3K are mediated through the activation of the downstream target Akt, a 57-kDa serine/threonine protein kinase, which belongs to the family of the AGC protein kinases (Hanada *et al.,* 2004). Most of the studies performed on PtdIns-dependent signal transduction pathways have focused on events that occur at the plasma membrane and in the cytoplasm. However, phosphoinositides and their biosynthetic machinery are also localized in the nucleus (Irvine 2004; Martelli *et al.,* 2005a; Manzoli *et al.,* 2005). Remarkably, nuclear inositol lipid cycle is largely independent from that of the plasma membrane, suggesting that the nucleus constitutes a functionally distinct compartment for PtdIns metabolism. PtdIns(3,4,5)P_3,

PI3K, and Akt have been reported to be present in the nucleus (Martelli *et al.,* 2006a). In this review article, we shall update our knowledge of the roles played by these molecules in the nucleus in relationship with carcinogenesis and anti-apoptotic signaling. However, we shall firstly review some general data about 3′-phosphorylated inositides, PI3K, and Akt.

3'-phosphorylated inositol lipids and PI3K

Resting mammalian cells contain significant levels of PtdIns(3)P, but hardly any of the other 3'-phosphoinositides. While the overall levels of PtdIns(3)P do almost not increase upon cell stimulation with agonists, the levels of the other 3'-phosphoinositides can rise dramatically (Vanhaesebroeck *et al.,* 2001). Since these lipids are not the target of any known phospholipases, they are metabolized by phosphatases that act on the inositol ring. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a 3′-phosphorylated inositol lipid-phosphatase which has received much attention recently, because of its role as a tumor suppressor gene (Sansal and Sellers, 2004). PTEN converts PtdIns(3,4)P_2 to PtdIns(4)P, and PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2. In a significant number of human cancers, PTEN is mutated and/or inactivated so that the PI3K signaling pathway is constitutively activated as a result of the high PtdIns(3,4,5)P_3 levels (Chow and Baker, 2006). Two other phosphatases, SHIP-1 and SHIP-2 (for Src Homology domain-containing Inositol Phosphatases), are capable of removing the 5-phosphate from PtdIns(3,4,5)P_3 to yield PtdIns (3,4,)P_2 (Backers *et al.,* 2003), but their role in down-regulating PI3K-dependent signals is not well understood, taking also into account that PtdIns (3,4,)P_2 shares several functions with PtdIns(3,4,5)P_3 (in addition to unique signalling properties) and may prolong the duration of PtdIns(3,4,5)P_3 signaling. There are multiple isoforms of PI3K in mammalian cells, and these are subdivided into three classes, referred to as I, II, and III (Vanhaesebroeck *et al.,* 2001). Our review will focus on class IA PI3Ks which are the most investigated because they are generally coupled to extracellular stimuli. They display a preference *in vivo* for PtdIns(4,5)P_2 as a substrate. Class IA PI3Ks are heterodimeric enzymes composed of a p110 catalytic subunit (α, β, and δ) and an adaptor/regulatory subunit. There are at least five adaptor proteins that are generated by expression and alternative splicing of three different genes (referred to as Pik3r1, Pik3r2, and Pik3r3). The regulatory subunits function as adaptors and act to localize PI3K to the plasma membrane by the interaction of their SH2 (Src homology) domains with phosphotyrosine residues in activated receptors. They also serve to stabilize p110 and to limit its activity.

Akt

At present, three members of the Akt family have been identified and are referred to as Akt1, Akt2, and Akt3. Although they are products of different genes, they are highly related exhibiting more than 80% sequence homology (Hanada *et al.,* 2004; Brazil *et al.,* 2004). In response to a variety of stimuli (hormones, growth factors, cytokines), inactive (cytosolic) Akt is recruited to the plasma membrane by the products of PI3K, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Then, Akt is phosphorylated at threonine 308 by a phophoinositide-dependent kinase 1 (PDK1), whose activity strictly depends on 3'-phosphorylated inositol lipids, (Mora *et al.,* 2004) and at serine 473 by a still undefined kinase. This double phosphorylation activates Akt (Brazil *et al.,* 2004). A plethora of Akt substrates have been identified and these include, among the others, BAD, Raf1, members of the FoxO family of transcriptions factors, Iκ-B kinase, procaspase-9, GSK-3-α/β, mTOR, cyclin D1, $p27^{KIP1}$, $p21^{CIP1}$ (Brazil *et al.,* 2004). The large variety of proteins that are phosphorylated by Akt explains why this kinase has rapidly emerged as a key mediator of cell proliferation, differentiation, and survival. Moreover, increasing evidence indicates that Akt plays an important role in tumorigenesis and resistance to chemotherapeutic drugs (Fresno Vara *et al.,* 2004; Martelli *et al.,* 2005b; Martelli *et al.,* 2006b).

Nuclear 3'-phosphorylated inositol lipids and class I_A PI3Ks

The presence of these inositol lipids in the nuclear compartment has been demonstrated by means of different techniques (radioisotope labeling, immunocytochemistry, quantitative immunogold electron microscopy) (Deleris *et al.,* 2006; Martelli *et al.,* 2006a; Lindsay *et al.,* 2006) in a variety of cell types, including PC12 rat pheochromocytoma, Saos-2 human osteosarcoma, rat hepatocytes, HepG2 human hepatocarcinoma, and HL60 human

promyelocytic leukemia (reviewed in Neri *et al.,* 2002). While control of cytoplasmic class IA PI3K is quite well defined, regulation of its nuclear counterpart has been unclear. A major breakthrough has been achieved in PC12 cells stimulated with NGF. By means of a yeast two-hybrid approach, Ye et al. (2000) identified the protein PIKE (Phospho-Inositide 3-Kinase Enhancer) as a novel physiological regulator of nuclear class I_A PI3K. PIKE is a nuclear GTPase characterized by a PX domain and three proline-rich domains, which typically bind to SH3 domains of target proteins. Retroviral infection of PC12 cells showed that NGF-induced nuclear PI3K activity was blocked by a dominant-negative form of PIKE, and that PI3K activation by PIKE was GTP-dependent and required the presence of both p85 and p110 subunit. Subsequently, the same group identified nuclear phosphoinositide-specific phospholipase C (PI-PLC) γ1 as the guanine nucleotide exchange factor (GEF) for PIKE (Ye *et al.,* 2002). Indeed, the SH3 domain of PI-PLCγ1 directly bound the third proline-rich domain (amino acids 353-362) of PIKE and this interaction stimulated GDP dissociation, markedly enhanced GTP binding to PIKE, and was required for nuclear PI3K activation. This finding might partly explain the previous puzzling observation that the mitogenic activity of PI-PLCγ1 does not actually require it to be catalytically active, but does indeed require the SH3 domain to be present (Bae *et al.,* 1998). In addition, the same authors have suggested that down-regulation of nuclear PI3K activity could result from the interaction between PIKE and the protein 4.1N (Ye *et al.,* 2000). Indeed, in NGF-treated PC12 cells, they observed protein 4.1N translocation to the nucleus with a slower time course than for PI3K translocation and PIKE activation. The binding of the protein 4.1N to PIKE inhibited PIKE GTPase activity and prevented association between PIKE and PI3K, resulting in nuclear PI3K activity decrease. The initially identified PIKE isoform is now referred to as PIKE-S (for Shorter), because two more PIKE isoforms have been subsequently identified, PIKE-L (Longer) and PIKE-A, which specifically binds to active Akt. While PIKE-S is exclusively localized in the nucleus, PIKE-L occurs both in the nucleus and cytoplasm. However, its function in the nucleus has not been clarified yet (Ye, 2005).

Nuclear Akt

It is now clear that phosphorylated (active) Akt is present within the nucleus. Indeed, some of its substrates are resident within this organelle, such as the FoxO family of transcription factors (Arden and Biggs, 2002) or the transcriptional coactivator p300 (Pekarsky *et al.,* 2000). Either Akt1 or Akt2 have been reported to migrate into the nucleus in response to a variety of stimuli including serum, activation of B-lymphocytes, hypoglicemic coma, mitogenic stimulation with polypeptide growth factors such as insulin-like growth factor-1 (IGF-1), differentiating treatment of PC12 cells with NGF, or exposure of HL60 and NB4 cells to retinoids (Neri *et al.,* 2002; Matkovic *et al.,* 2006). The nuclear localization signal (NLS) motif of Akt has not been identified so far, nevertheless the oncogene Tcl1 may be involved in Akt nuclear localization (Pekarsky *et al.,* 2000). Whether Akt may be phosphorylated and activated within the nucleus, is controversial. There are reports showing that Akt did not require phosphorylation for entering the nucleus (e.g. Saji *et al.,* 2005). Even though PDK1 has been identified in the nucleus (Kikani *et al.,* 2005), several lines of evidence suggest that Akt migrates to the nucleus after having been phosphorylated at the plasma membrane and that nuclear PDK1 does not target Akt. Rather, it seems that PDK1 nuclear translocation may be a mechanism to sequestrate it from activation of cytosolic signaling pathways (Lim *et al.,* 2003). Indeed, a recent report has demonstrated that in NGF-stimulated PC12 cells Akt phosphorylation is essential for nuclear translocation and retention (Xuan Nguyen *et al.,* 2006). There exists quite an ample body of literature on the localization of active Akt in the nucleus of neoplastic cells. The presence of nuclear phosphorylated Akt has been reported in lung, breast, prostate, and thyroid cancers, as well as in acute myeloid leukemia blasts (Lee *et al.,* 2002; Nicholson *et al.,* 2003; Van de Sande *et al.,* 2005; Vasko *et al.,* 2004; Brandts *et al.,* 2005; Montironi *et al.,* 2005). It is intriguing that in the prostate, the extent of Akt nuclear localization increases during the progression from normal tissue to low grade prostatic intraepithelial neoplasia (PIN), high grade PIN, and tumor (Van de Sande *et al.,* 2005). Furthermore, in prostatic carcinomas the extent of Akt nuclear localization correlates with the Gleason score, which is the most powerful predictor of tumor progression after prostatectomy

(Montironi *et al.,* 2005). All three Akt isoforms display a classic leucine rich, leptomycin-sensitive nuclear export sequence (NES). Stable overexpression of Akt1 with a non-functional NES, resulted in persistent nuclear localization of Akt1 and enhanced cell migration *in vitro* of Akt1$^{-/-}$ fibroblasts (Saji *et al.,* 2005). This finding may further support the hypothesis that Akt nuclear localization is somehow involved in some aspects of carcinogenesis and/or tumor progression.

Nuclear PTEN

There are several reports which have addressed the issue of nuclear PTEN. Four, non-traditional, putative NLS motifs have been identified in PTEN. Mutations in each of the four NLS-like region of PTEN did not alter entry into the nucleus. However, when mutations were combined, it was found that nuclear localization of PTEN was affected, thereby indicating that nuclear import requires two NLS-like motifs acting in concert. Double NLS mutants did not interact with the major vault protein (MVP), a previously hypothesized nuclear-cytoplasmic transport protein (Chung *et al.,* 2005). Consistently with this hypothesis, down-regulation of MVP decreased the nuclear localization of PTEN (Minaguchi *et al.,* 2006). Recently, however, it has been suggested that PTEN enters the nucleus by a Ran GTPase-dependent mechanism (Gil *et al.,* 2006). In contrast, others, have claimed that PTEN enters the nucleus mainly by diffusion (Liu *et al.,* 2005). Whichever the case, there is general consensus over the fact that a decrease in nuclear PTEN characterizes several types of human neoplasia, including thyroid carcinoma and melanoma (Gimm *et al.,* 2000; Whiteman *et al.,* 2002). Interestingly, in MCF-7 breast cancer cells, intranuclear PTEN levels correlate with the cell cycle, with the highest levels being observed at/or before G0-G1. Therefore, it has been suggested that nuclear PTEN could help coordinate cell cycle arrest (Ginn-Pease and Eng, 2002). This could be achieved through a down-regulation of cyclin D1 and involved a specific down-modulation of MAP kinase by nuclear localized PTEN (Chung *et al.,* 2006). Interestingly, NGF-mediated differentiation of PC12 cells (which associates with reduced cell proliferation) is characterized by increased levels of nuclear PTEN (Lachyankar *et al.,* 2000). Furthermore, nuclear PTEN alone is capable of suppressing anchorage-independent growth of U251 MG cells without inhibiting Akt activity. Growth suppression induced by nuclear PTEN is dependent on possessing a functional lipid phosphatase domain (Liu *et al.,* 2005). Therefore, it seems plausible that this effect of PTEN is related to a decrease in intranuclear 3′-phosphorylated inositol lipid mass, and not to its protein phosphatase activity. Nevertheless, others have shown that intranuclear PtdIns(3,4,5)P_3 levels are insensitive to PTEN expression in the nucleus (Lindsay *et al.,* 2006). Catalytically active nuclear PTEN enhanced cell apoptotic responses (Gil *et al.,* 2006) and this effect could be in relationship with the observation that nuclear PTEN forms a complex with p300 and plays a role in maintenance of high p53 acetylation in response to DNA damage thus regulating the p53 levels (Li *et al.,* 2006). As for Akt, an interesting correlation between PTEN nuclear localization and cell proliferation/differentiation and transformation has begun to take shape. Indeed, PTEN usually localizes to the nucleus of primary normal cells. For example, thyroid follicular cells, normal melanocytes, and pancreatic islet cells express PTEN prodominantly in the nucleus, whereas thyroid carcinomas, melanomas, and endocrine pancreatic tumors show a dramatic reduction in PTEN nuclear staining (Gimm *et al.,* 2000; Whitman *et al.,* 2002; Perren *et al.,* 2000). Interestingly, in follicular thyroid tumors, the intranuclear PTEN levels are inversely correlated to the localization of Akt: while nuclear PTEN diminishes during the progression from normal tissue to adenoma to carcinoma, the amount of phosphorylated Akt within the nucleus increases (Vasko *et al.,* 2004). Nevertheless, it remains to be established whether this findings could be related to a PtdIns(3,4,5)P_3-dependent phosphorylation of Akt which takes place inside the nucleus.

Involvement of 3'-phosphorylated inositol lipid metabolism and Akt in NGF-dependent anti-apoptotic signaling of PC12 cells

PI3K/Akt pathway is by far the most important signaling network for cell survival. Traditionally, anti-apoptotic signaling by PI3K/Akt has been thought to take place at the plasma membrane level and in the cytoplasm (Franke *et al.,* 2003). However, recent findings point to the likelihood that nuclear PI3K plays an essential role in promoting cell survival also through nuclear PtdIns (3,4,5)P_3 synthesis (Ye, 2006). PI3K migrates to the PC12 cell

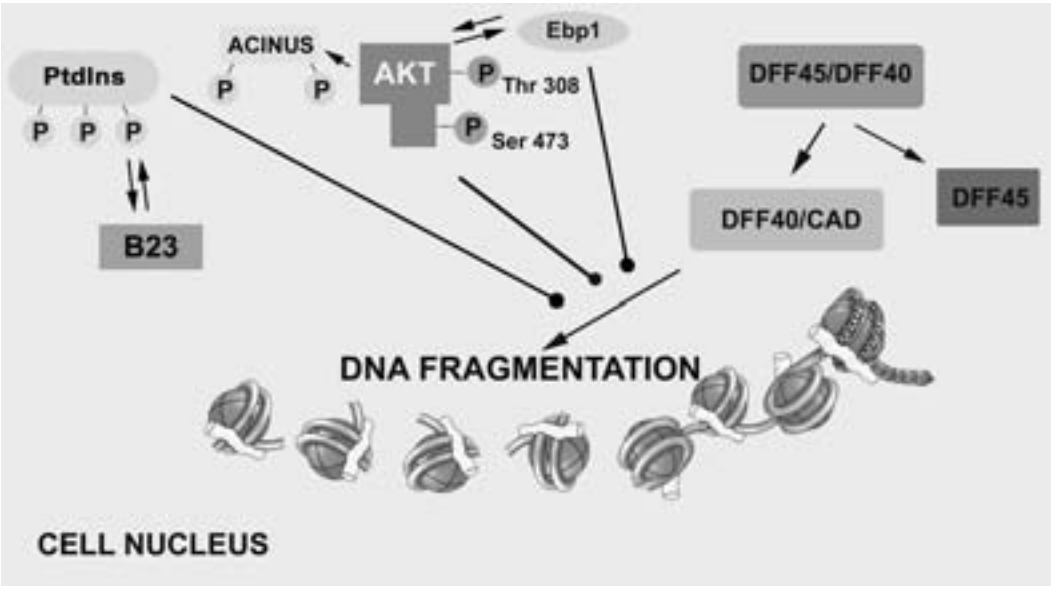

Figure 1. Schematic diagram showing the relationship between PtdIns (3)P, activated Akt and DNA fragmentation inside the nucleus. The pathways depicted hint at the anti-apoptotic role of this signalling cascade.

nucleus in response to NGF (Neri *et al.,* 1999). Taking advantage of a cell-free system, it has been shown that nuclei isolated from NGF-treated PC12 cells were resistant to DNA fragmentation factor/caspase activated DNase (DFF40/CAD) - dependent DNA cleavage initiated *in vitro* by activated cell-free apoptotic solution, consisting of HEK293 cell cytosol supplemented with purified active caspase-3 (Ahn *et al.,* 2004). Nuclei from constitutively active PI3K adenovirus-infected cells displayed the same resistance as those treated with NGF, whereas PI3K pharmacological inhibitors, immunodepletion of PI3K from nuclear extracts with anti-p110 antibody, and dominant negative PI3K or PIKE abolished it. PtdIns (3,4,5)P_3 alone, but not PtdIns (3,4)P_2, PtdIns (4,5)P_2 or PtdIns (3)P, mimicked the anti-apoptotic effect of NGF. The involvement of nuclear PtdIns (3,4,5)P_3 in the protecting role of NGF was also substantiated by an experiment in which isolated nuclei were preincubated with PTEN and then analyzed for DNA fragmentation. It was found that PTEN pre-treatment abolished the protective effect of NGF, even though it was not demonstrated that PTEN actually decreased the amount of nuclear PtdIns (3,4,5)P_3 (Ahn *et al.,* 2004). Since NGF treatment stimulates migration of phosphorylated Akt to the nucleus of PC12 cells (Borgatti *et al.,* 2003), the role of nuclear Akt in the anti-apoptotic action of NGF was also examined. It turned out that nuclei isolated from cells overexpressing wild type or constitutively active Akt were resistant to internucleosomal DNA cleavage, whereas those from dominant-negative Akt-infected cells showed DNA cleavage in spite of NGF treatment, thus demonstrating that nuclear Akt is required for NGF-mediated anti-apoptotic signaling (Figure 1). Nevertheless, in the absence of NGF treatment, all the nuclei displayed DNA degradation, suggesting that Akt activation alone is not sufficient to inhibit DNA cleavage (Ahn *et al.,* 2004). The same group identified protein B23/nucleophosmin as a receptor for nuclear PtdIns (3,4,5)P_3. Indeed, depletion of B23 from nuclear extracts or B23 knockdown abolished NGF-dependent protective effect in PC12 cells, whereas overexpression of B23 prevented apoptosis (Ahn *et al.,* 2005). Protein B23 directly interacts with and inhibits active CAD in a PtdIns (3,4,5)P_3-dependent fashion. As to anti-apoptotic action of nuclear Akt, it has been recently shown that Akt phosphorylates acinus on Ser 422 and 573, resulting in its resistance to caspase-dependent cleavage and inhibition of acinus mediated chromatin condensation (Hu *et al.,* 2005). Acinus, which induces apoptotic chromatin condensation after cleavage by caspase-3 without inducing DNA fragmentation is essential for apoptotic chromatin condensation *in vitro* and *in vivo* (Sahara *et al.,* 1999). Furthermore, nuclear Akt prevents DNA fragmentation by CAD through its association with protein kinase C-phosphorylated p48 isoform of nucleolar protein Ebp1 (Figure 1) (Ahn *et al.,* 2006).

Conclusions

As is clear from this overview, nuclear PI3K, PtdIns(3,4,5)P_3, Akt, and PTEN may be involved in key cellular processes, including carcinogenesis and apoptosis protection. While our knowledge of how this signaling cascade could result in neoplastic transformation is virtually non-existent, we understand more about its involvement in blocking apoptosis. A challenge for the future will be to better elucidate the anti-apoptotic functions of nuclear PI3K/ PtdIns (3,4,5)P_3/Akt/PTEN signaling. For example, we do not know whether or not this system is operative only in neural cells (Ye, 2005) or also in other cell types, including hepatocytes and cardiomyocytes, as preliminary evidence would suggest (Martelli *et al.,* 2006a). A central question is whether this pathway is also activated by other neurotrophins which protects neural cells from apoptosis, such as IGF-1. Identification of additional targets and/or interacting partners within the nucleus will be of outmost importance for a better comprehension of the roles played by this signal transduction system. Furthermore, it should not be forgotten that nuclear PI3K seems to be critically involved in processes other than tumorigenesis and apoptosis, such as myeloid cell differentiation (Bertagnolo *et al.,* 2004). However, further elucidation of this complex and peculiar nuclear signaling pathway is expected to provide new potential targets for pharmacological interventions in major human diseases, including cancer and degenerative disorders in which inappropriate apoptosis is thought to play a fundamental role, such as heart failure, Parkinson's disease, and amyotrophic lateral sclerosis.

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Neuroendocrine regulation and tumor immunity

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The morphogenetic events leading to the transendothelial passage of lymphoid and tumoral cells are analyzed in light of a very recent and global theory of intercellular communication designated as the Triune Information Network (TIN). The TIN system is based on the assumption that cell-cell interactions primarily occur through cell surface informations or topobiological processes, whose mechanisms rely upon expression of adhesion molecules, and are regulated by an array of *locally-borne* (autocrine/paracrine signals and autonomic inputs) and *distantly-borne* (endocrine secretions) messages. The final aim of the TIN is to control homeostatic functions crucial for the organism survival, like morphogenesis. Knowledge of the TIN signals involved in lymphoid and tumoral cell intravasation might offer a new perspetive to study the mechanisms of tumor immunity. Recognition of tumor target cells by immune cytotoxic effectors, in fact, can be considered a notable case of TIN-mediated cell to cell interaction. In particular, Natural Killer (NK) cells play a role in the cell-mediated control of tumor growth and metastatic spreading. Cell targeting and killing are dependent on the different NK cell receptors and on the efficacy of NK cells after cytokine and monoclonal antibody administration in cancer therapy. Since efficacy of NK cell-based immunotheraphy has been proven in KIR-mismatch regimens or in TRAIL-dependent apoptosis, the ability to manipulate the balance of activating and inhibitory receptors on NK cells and of their cognate ligands as well as the sensitivity of tumor cells to apoptosis, opens new perspectives for NK cell based immunotherapy.

Key words: Topobiology, paracrine secretion, natural killer cells, cytokines, cytotoxicity, tumor immunity, cell activation.

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From an evolutionary and developmental perspective the cell-cell interactions occurring during the transendothelial passage of lymphocytes (see Azzali G, this issue) may be seen as a *local* morphogenetic event, based on cell surface interactions or topobiological processes (Toni 2004a). Both lymphocytes and endothelial cells, in fact, undergo substantial reorganization of cell shape during intravasation, primarily following cell surface contact, suggesting that cell adhesion molecules, substrate adhesion molecules, and cell junctional molecules must be called into play during this phenomenon (Toni 2003). In addition, some common mesodermal origin of mononuclear and endothelial cells suggests that they may share a modality of *reciprocal recognition* (Toni 2004a). Indeed, endothelial/vascular precursors have been isolated in humans from the mononuclear fraction of peripheral blood CD34, Flk-1, AC133 and Tie2 antigen-positive cells, both in basal state and after granulocyte-colony stimulating factor treatment of donors, to favour mobilization of $CD34^+$ elements from bone marrow to peripheral blood (Asahara 1997). Since specific growth factors may selectively address these mesodermal progenitors towards either a mononuclear or an endothelial differentiation lineage (Ishikawa 2004), it is likely that an array of paracrine signals common to both cell types may constitute an ideal *niche* for their interaction (Fuchs 2004). In addition, the possibility that the endothelial canalization is triggered also by different phenotypes of neoplastic cells (see Azzali G., this issue) suggests that the repertoire of repricocal extracellular signals must be an heritage common to many different cell lineages. This raises the possibility that transendothelial passage is a special case of a more general mechanism regulating intercellular communication. Very recently a global theory of intercellular communication has been proposed, including the paracrine signals as a part of a hierachically-ordered, informational supersystem of

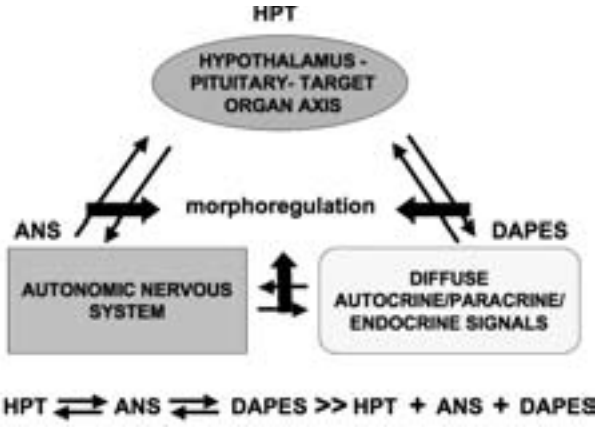

Figure 1. Schematic organization of the Triune Information Network (TIN) system. TIN molecules may be produced by any cell in the vertebrate organism, as a response to specific physiological and pathophysiological conditions. They ensure a constant dialogue between the hypothalamic-pituitary-target organ axis (HPT), the neurons of the autonomic nervous system (ANS) and those secretory elements scattered throughout body compartment (diffuse autocrine/paracrine/endocrine signals or DAPES). Such a *triangular* communication rises from extracellular messages developing hierarchically during phylogenesis, from invertebrates to vertebrates and man. The continuous interaction between the various TIN signals yields effects larger than the sum of each of those deriving from any single structure of origin (this result is depicted in the equation at the bottom of the figure). As a result, the final aim of this informational supersystem is to provide control of basic homeostatic functions, including morphoregulation. At least part of these actions can be achieved by either modulation of DNA methylation patterns or heterodimerization of transcription factors for morphoregulatory genes, like those of adhesion molecules (from Toni 2004b, with permission, partly modified).

internal secretions. By analogy with the famous acronym coined by Paul D. MacLean for the evolutionary meaning of a hierarchically-organized brain superstructure, this supersystem has been designated as the Triune Information Network or TIN (Toni 2004b, Ravera 2005) (Figure 1). Thus, knowledge of the TIN might critically contribute to clarify the signal machinery regulating cellular intravastion.

The Triune Information Network system

The TIN system rises progressively during evolution in invertebrates and diffuses to a growing number of body structures in vertebrates, resulting able to control homeostatic functions fundamental for survival, like morphogenesis (Toni 2004b). In a sense this network ricapitulates, at least in part, the classical neuroendocrine system (NES). However, primarily in mammals and man, the TIN encompasses not only the classic amine precursor uptake and decarboxilation or APUD system (Pearse 1986) but also the hypothalamic-pituitary-target organ system, the autonomic nervous system, the immune system and any other body system performing internal secretory outputs. Indeed, it is now clear that cells residing in any part of the vertebrate body, including those of the immune system and endothelia, may express functional properties originally ascribed only to neurons of the central nervous system and classical endocrine glands (Toni 2004b). Specifically, the ability to synthesize and secrete amine hormone/transmitters and peptide hormone/transmitters, as well as the presence of markers of neural determination, like the enzyme neuron specific enolase and the acidic proteins chromogranins (DeLellis 1991). Immune cells, in particular, are capable of producing peptides, amines and growth factors which can act as either hormones, neurotransmitters or local tissue regulators (Toni 2004b), as well as may establish *synaptic-like* contacts between them and with other cell types (Vitale 2007). Although this capacity is still named as neuroendocrine function, it may also be found in any tissue type after environmental challange, including inflammation, trauma and neoplasia (Toni 2004b). Consequently, we may no longer assume, as originally proposed by A.G.E. Pearse (Pearse 1986), that the presence of this function means existence of a neural crest-derived, committed neuroendocrine precursor. More simply, it may be explained by the presence of uncommitted stem cells with multidirectional differentiation phenotypes, each able to express a peculiar anatomical identity meanwhile sharing a common system of extracellular signals (DeLellis 1991).

In light of the TIN theory, it is now possibile to predict that analysis of molecular events regulating the transendothelial passage need to take into account the role of autocrine, paracrine, endocrine and autonomic inputs to both mononuclear, tumoral and endothelial elements participating to the endothelial canalization. Even small differences in homeostatic settings and environmental challanges, in fact, are expected to yield substantial modifications to the time-course and morphologic features of the intravasation process. Similarly, it would have no sense to analyze the intracellular signalling chain active during lymphocyte-endothelium or tumoral cell-endothelium re-shaping irrespective of the three-dimensional (3D) *geometry* of interacting cells. As a result, *in vitro* evaluation in a standard bidimensional tissue co-culture might lead misleading informations. In contrast, the recent proposal for *ex situ* 3D co-culturing of endothelial and

epithelial progenitors on biocompatible scaffolds (Toni 2007) could offer a new perspetive. In such a frame, in fact, it would be possible to analyze the TIN signals regulatiing the 3D arrangement of lymphocytes and neoplastic cells during both their transendothelial passage and reciprocal recognition, like in the case of Natural Killer cells actions.

Natural killer cells and tumor immunity

Natural Killer (NK) cells represent the 10-20% of peripheral blood mononuclear cells, but they can be also present in lymph nodes, spleen and bone marrow, and can be induced to migrate towards inflammation sites by different chemoattractants. NK cells are able to kill target cells by a lytic machinery in an activation-independent way, suggesting a role in the control of tumor growth. NK cells are not an homogenous population. In fact, they express CD56 at different levels (dim or bright) and also the CD16 antigen (Ag) can be present or not on their surface. $CD56^{bright}$ NK cells have been recently defined as the *cytokine responsive* NK subset that may not require *licensing* by host MHC-I molecules (Anfossi 2006). NK cells express on their surface both inhibitory and activatory receptors (Bottino 2004). The several types of inhibitory receptors show different specificities for alleles of class I molecule. In particular, the killer Ig-like receptors (KIRs) bind HLA-class I, and the heterodimeric receptors CD94-NKG2A/B recognize HLA-E (Braud 1998). Cancer cells frequently lack a MHC-I allele, and therefore are susceptible to NK cell lysis. In the absence of inhibitory signals, NK cell cytotoxicity must however be activated by a set of triggering receptors. Spontaneous cytotoxic activity is mainly triggered by NKG2D, leukocyte adhesion molecule DNAM-1 (CD226), and Natural cytotoxicity receptors (NCRs), while CD16, by binding the Fc portion of IgG, binds to opsonized cells mediating antibody dependent cellular cytotoxicity (ADCC) (Moretta 2004). NKG2D and DNAM-1 recognize stress-induced ligands expressed by several tumor cell lines, while NCRs mediate cell lysis of many cancer cells.

Upon cytokine stimulation, NK cells become lymphokine activated killer cells (LAK) that proliferate, produce cytokines and up-regulate effector molecules such as adhesion molecules, perforin, granzymes, FasL and TRAIL (Figure 2). LAK cells became able to induce perforin/granzymes-dependent necrosis of target cell and TNF ligand family members-induced apoptosis of the target cell. Given the ability of TRAIL to kill many cancer cell types, while sparing normal tissues, the use of recombinant TRAIL has been proposed in clinical trials (Smyth 2003). TRAIL is present in the BM, a site of NK cell as well as erythro-myeloid differentiation. Since it has been demonstrated that erythroid cell differentiation is affected *in vitro* and *in vivo* by recombinant TRAIL (Zamai 2000, Mirandola 2006, Ashkenazi 1999), its use in therapy should be cautious. Activated NK cells themselves express different death receptors, such as TRAIL-R2 and CD95, that are generally seen as implicated in the termination of NK cell response and in tumor responses to specific immune activities (immune counterattacks). However, differently from erythro-myeloid cells, NK cells are usually protected from TRAIL-induced apoptosis thanks to cytokine-dependent c-FLIP induction (Mirandola 2004).

Among the activatory cytokines, IL-15 is believed to be responsible for NK cell development *in vivo*, and is a survival factor that protects lymphocytes from IL-2-activation-induced cell death (AICD). Recent evidences suggest a nonreduntant unique role for IL-15 in the differentiation, proliferation, survival and activation of natural killer (NK) cells (Rodella 2001). IL-2 acts as growth factor for NK cell progenitors and mature NK cells, and induces the production of NK effector molecules, enhancing NK lytic activity. IL-12 and IL-18, NK activatory cytokines active during late NK cell differentiation, have been demonstrated to synergistically enhance cytotoxicity against tumor targets and IFN-γ production by NK cells (Golab 2000). IFN-γ induces type 1 immune response and directly acts on cancer cells. Finally, IL-21, another cytokine binding the common γ chain (shared with IL-2, IL-4, IL-7, IL-9 and IL-15), has been demonstrated to favour the onset of the most cytotoxic $CD56^{dim}CD16^{+}$ NK cell subset and to enhance its cytotoxicity (Parrish-Novak 2000).

IL-2 activated NK cells were used in clinical trials for the treatment of solid primary or metastatized cancers (Rosenberg 1993). Subcutaneous injections of NK-stimulating doses of IL-2 or administration of pre-activated NK cells (adoptive transfer of LAK cells), showed a 15-30% positive effects in patients with advanced renal cell carcinoma (RCC) or melanoma (MEL) (Rosenberg 1993). Unfortunately, IL-2 treatment is associated with life-threatening toxicity, essentially represented by

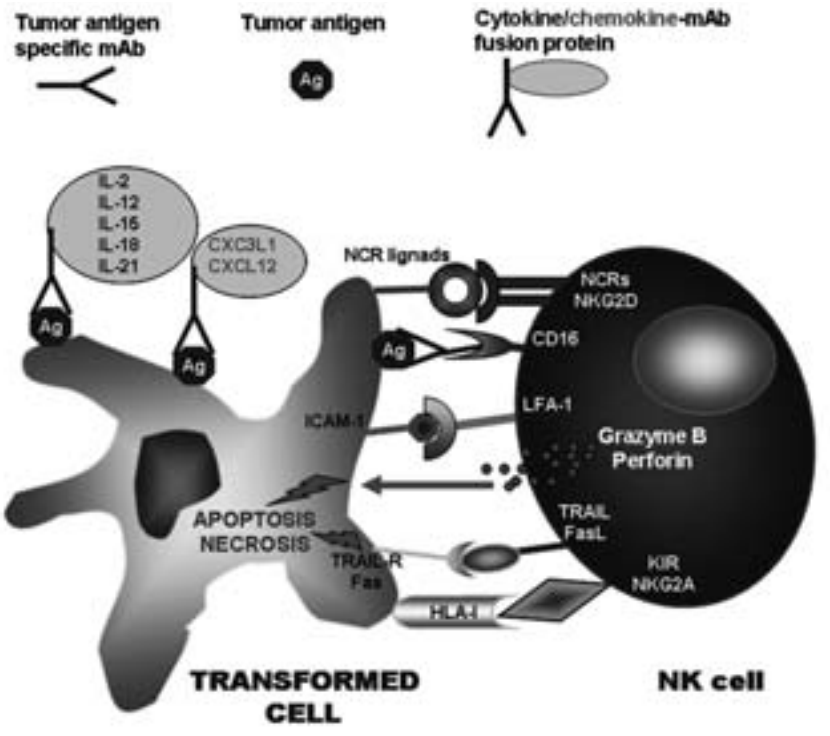

Figure 2. Scheme of the interaction between NK cell and target cell.

capillary leak syndrome. Another limitation of this approach is the fact that IL-2, but not IL-15, activated NK cells increase their sensitivity to apoptosis when in contact to vascular endothelium (Rodella 2001), likely causing a decrease in NK cell migration towards the cancer area. IL-15 would appear more efficient than IL-2 in expanding the NK cell compartment since it promotes the survival of NK cells, and protects from AICD. Unfortunately, extremely high doses of IL-15 are required to observe anti-tumor effects *in vivo*. Alternatively, early acting cytokines such as stem cell factor (SCF) have been used to enhance NK antitumor activity.

Differently from IL-2 and IL-15, IL-12 mainly enhances NK cell-mediated IFN-γ production, and IL-1 and IL-18 potentiate the effect of IL-12 by up-regulating the IL-12Rs expression on NK cells (Trinchieri 2003, Moretta 2006). Only mature NK cells can produce IFN-γ, while immature NK cells produce type 2 cytokines. The IFN-γ-induced type 1 immune responses as well as the terminal differentiation of NK cells therefore appear relevant to an effective antitumor activity. To this regard, IL-21, a promising cytokine able to build up NK cell antitumor acitivity (Nakano 2006), has been found to promote both the expression of genes associated with type 1 response and the terminal differentiation of the highly cytotoxic $CD56^{dim}/CD16^{+}$ NK cell subset which can potentially direct ADCC against tumor cells via CD16-Fc ligation (Strengell 2002). NK cell mediated ADCC response against tumor targets can be promoted by administration of monoclonal antibodies (mAbs) to tumor-associated Ags, a mechanisms of action that does not produce crossresistance or overlapping toxicities with conventional agents (Caligiuri 2004), and that can therefore be combined with cytokine-based immunotherapies.

Strategies that utilize NK cell donors mismatched for inhibitory NK receptors and MHC-I ligands, present in some allogeneic settings, have been more successful. An important antitumor role for alloreactive NK cells has been shown in patients with acute myeloid leukemia either after stem cell transplantation or adoptive tranfer of haploidentical NK cells (Ruggeri 2002). Donor NK cells attack host hematopoietic cells, but not other tissue. Thus, allogeneic stem cell transplantation or adoptive transfer of polyclonal or clonal NK cells with mismatch NK inhibitory receptors and HLA class I ligands, would produce graft-versus-leukemia (GvH) in the

absence of graft-versus-host desease (GvHD). The signals transduced by MHC-I inhibitory receptors become superfluous and likely exploited by some tumor cells to elude NK immunosurveillance. TNF-receptor mediated apoptosis of sensitive tumor cells should be NKR-independent, suggesting that this mechanism should however work upon NK cell activation, independently from the KIR/MHC-I setting. Mouse models of leukemia have demonstrated efficacy of anti-KIR blocking antibodies without adverse effects on normal cells, indicating the feasibility of treatments with antibody fragments to prevent KIR/NKG2A-MHC-I interactions in cancer therapy (Koh 2001) (Figure 2).

Conclusions

Interactions between solid tumor cells and the microenvironment *in vivo* create a context that promotes tumor growth, selection and protection from immune attack, suggesting that the tridimensional architecture of solid cancer lesions is likely one of the tumor mechanisms to escape immunosurveillance. To this regard, another important mechanism to control NK cell activity is their ability to traffic to tumor sites. Chemokines are key regulators of NK cell migration and are required to drive NK cells to tumor sites. NK cells express chemokine receptors on the cell surface and migrate vigorously in response to CXCL12 and CXC3L1 (Robertson 2002).

Finally, both conventional therapies and immunotherapy kill tumor cells inducing programmed cell death, thus selection of tumor cells resistant to apoptosis would be the reason of cross-resistance of cancer cells to chemotherapy and immunotherapy. Therefore, sensitization of tumor cells to activated cytotoxic lymphocytes by up-regulating either TNF family death receptors or effector activating ligands on tumor cells combined with immunotherapy have been pursued in order to overcome tumor cell resistance and establish an effective antitumor response. Today, the potential ability to manipulate not only the balance of activating and inhibitory receptors on NK cells but also their cognate ligands as well as the sensitivity of tumor cells to apoptosis opens new perspectives in NK cell based immunotherapy. Thus, detailed knowlede of the humoral environment involved, like that expected on the basis of the TIN system theory, could become critical to design any future *intelligent*, cell-mediated antitumoral therapy.

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