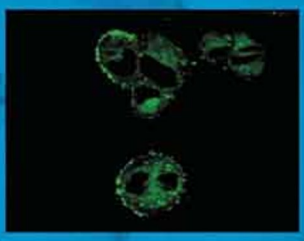
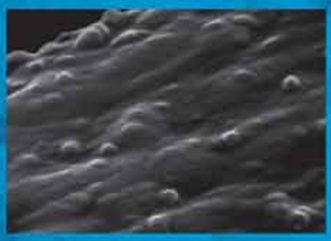
UPDATES in SURGERY

Alfonso Barbarisi
In collaboration with P. Bechi
P. Innocenti • C.A. Redi • F. Rosso

Biotechnology in Surgery


Springer

Updates in Surgery

Alfonso Barbarisi (Ed.)

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In collaboration with
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Foreword by
Enrico De Antoni

Springer

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To my patients,
To Alberto, Anna, Titty,
To Caterina,
To Alberto, Manlio, Anna,
with gratitude and love.

Foreword

The Italian Society of Surgery has always had a great interest in biotechnological developments which provide new and appropriate solutions to current surgical problems.

In the last decade, at the annual Congresses, the Society has always aimed at treating biotechnological subjects in the knowledge that it will bring improvements to surgery in the 21st century, as was the case with technological development in the last century.

It is with great pleasure and interest that the Italian Society of Surgery offers its members and the whole medical community the opportunity to approach and to expand this branch of human knowledge for new applications in health care.

We therefore appreciate the efforts of our member Alfonso Barbarisi, President of the European Surgery Society (ESS) and Head of the Surgical Department of the Medical School at the Second University of Naples. Prof. Barbarisi has carried out research both in Italy and abroad for a long time with persistance, belief and philosophic attitude**.** The purpose of this work is to offer surgeons an approach to biotechnological techniques for their better use in every day surgical activity. The other goal is to attract biotechnologists to the complex query for *help* by medicine in the attempt to solve mankind's health needs in the best way possible.

I am convinced this book has a great scientific and practical value. It will make surgeons more conscious both in applying biotechnologies already in use and in more rapidly taking on future biotechnological discoveries in their culture. I am sure it will be accepted with enthusiasm by the Italian and international surgical community.

Rome, October 2010

Enrico De Antoni
President, Italian Society of Surgery

Preface

The 20th century has drawn to a close, the century when surgery took huge steps forward thanks to the progress of technologies. Now we are approaching the century of biotechnologies which will cause not only a progress in surgery, but a real *cultural revolution* which will completely change the approach to solving different problems in medicine. The aim of this book is to bring surgeons nearer to biotechnologies and to overcome the cultural gap dividing them.

A lot of biotechnologies have already been put forward and are used as a routine in surgical practice, to the complete unawareness of surgeons. It is important that surgeons realize this and understand the real mechanism of biotechnologies and their practical applicability, so they can be more aware of them and master their use.

A surgeon has always had a blinkered view of patient treatment because he has always suffered from the limits of his instruments. He has always been a technologist, in the sense that since the beginning of surgery he has always needed technology, from the scalpel to advanced surgical instruments. As a consequence he has always been a protagonist of technological progress, he has invented new surgical instruments, he has always modified them, he has used physical means (*iron* and *fire*); he has always cooperated with technologists.

In this new century, the first of the new millennium, an increasing amount of knowledge which is unusual to the surgeon's traditional technological training is encroaching upon surgery, hence the aim of this book. Now it is urgent to bring surgeons towards this knowledge (biotechnology) which by its nature is completely different from the technologies used so far, because it goes beyond the senses of sight and touch, which have been till now the essence of the surgeon's action.

The cellular and molecular dimension of biotechnologies is really by far the most advanced and modern surgical action. The use of bio-materials produced biotechnologically, the use of engineering tissues, the use of biomarkers is a routine that is not near the traditional action of surgeons who have a sort of distrust which lies not in a refusal of the new, but in poor knowledge of biotechnologies.

A solid, workable and cultural alliance between biotechnologies and surgeons is required, as has already happened in the past between surgeons and technologies.

A common language between surgeons and biotechnologists will bring further, revolutionary progress to surgical sciences in the 21st century, which is purported to be the century of biotechnologies.

Naples, October 2010 **Alfonso Barbarisi**

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The Philosophy of Biotechnology

1

A. Barbarisi

1.1 Introduction

Biotechnologies are those technologies living organisms use to keep themselves alive and which, once discovered, human beings use to make products useful to them. Biotechnologies are at the base of the phenomenon of *life* and so they are naturally present in nature. They are not invented by man, they are instead discovered while studying the phenomenon of *life*. So biotechnologies differ from technologies. Technologies are the result of man's intellect and creativity, which are used to invent machines and devices not present in nature, but produced to satisfy man's life needs.

The use of biotechnologies takes place using living organisms or parts of them to make large quantities of products useful to man, and this is one of the areas of high technology in great expansion and from which very interesting results for productive activities have come and are expected.

Many people think biotechnologies are recent developments, but in fact they have always existed and they have been used for thousands of years. Today, however, the huge progress in the knowledge of vital mechanisms at the cellular, sub-cellular and molecular level has made it possible to recognize and discover them in their minimal mechanisms and to take advantage of this knowledge widely and with great flexibility.

Biotechnologies have been traditionally used in productive activities, in agriculture, in zootechnics and in food production in general for a long time. In prehistoric times man prepared drinks and fermented food: the Sumerians and the Babylonians produced wine and beer as early as 6000 B.C. and the Egyptians produced leavened

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bread as early as 4000 B.C. In reality these forefathers of biotechnology were completely unaware of the processes taking place during the achievement of these products and they did not realize that the action of living organisms was used in them. It was only thanks to Leeuvenhoek's microscope that researchers managed to observe the microorganisms responsible for the production of beer and leavened bread. And only Pasteur, between 1857 and 1876, understood the general mechanisms of beer production and identified the mechanisms making it possible.

After the discovery of a new substance produced by microorganisms – penicillin – Fleming laid a milestone with the discovery of a new class of drugs: antibiotics. However, the ability to produce antibiotics in large quantities needed another biotechnological passage: the selection of a microbial species able to produce these substances in greater quantities than the species found in nature.

In modern times, the development of biotechnologies has become extremely rapid. Their fast and widespread use has caused, both in their applications and in the same fundamental research, considerable and inevitable ethical problems regarding the protection of biodiversity and the validation of the biotechnological product.

One of the pivotal points of the new philosophy of the current knowledge on biotechnology is to let clinicians, surgeons and all its most recent users approach and face the problems and the requests of today's medicine and surgery. The main question is not to know the numerous biotechnological discoveries, but rather to understand their original application to the problems of medicine and surgery. This approach tries to show application solutions to the use of biotechnologies.

At present new technologies producing new vaccines or improving existing ones are available. Monoclonal antibodies are another biotechnology achievement which have found particular application in prevention, diagnoses and therapy thanks to their pureness, specificity and unlimited disposal. Gene therapy is another biotechnology application. This term refers to the transfer of genetic material to prevent or treat a disease. In the case of genetic diseases, for example, where a gene is dysfunctional or absent, this therapy allows the transfer of the *right* gene to the sick organism, so as to correct the defect. By using the same gene manipulating techniques used in plants, we have discovered we can intervene even on the DNA of more complex organisms, from bacteria to superior mammals, with the creation of transgenic animals. These animals are used in different areas: in the biomedical field, for example, they produce man's life-saving drugs in their milk, or they form the basis of experimental models which are indispensable in acquiring knowledge of very severe diseases. In the medical-surgical field investors are also concentrating their attention on the realization of transgenic animals for xenon-transplantations.

Another very current biotechnological application is in the field of stem cells. Man's body tissues are composed of different cells, each one with its own specific characteristics. However all of them derive from the primordial cell coming from the fusion of the male and female gametes during fertilization. At the very beginning of embryo development, each cell is totipotent because it can give rise to any of the 254 different cellular types present in an adult organism.

Tissue engineering is an emergent interdisciplinary research field which applies the methodologies and the techniques typical of material engineering (technologies)

and life sciences (biotechnologies) to understand and solve the problems linked to tissue repair and substitution in living organisms (prosthetic-substitution surgery).

The major improvement in the knowledge of the structure-function relations in biotechnology and in medicine and the recent developments obtained in chemical, material and bioengineering have in fact made possible the design of substitute functional tissues. This approach uses living cells associated with biomaterials in which, or on which, cells can proliferate, organize, and differentiate themselves in a manner similar to native tissue and thus three-dimensional organized tissues similar to the natural element to be rebuilt can be obtained. A new frontier in this field is the development of transplantable organs.

However, I think that in addition to the improvement of biotechnological knowledge, an appropriate philosophy of this knowledge is the strong trend in the use of biotechnology, rather than simply using technology for the solution of health problems.

Man has always tried to produce technologies to *subdue* nature and to defend himself from its adverse manifestations. This has given life to a competition which apart from being a failure has created a *vulnus* in the natural organization of things both in general and in the human organism.

The use of phenomena and of natural vital mechanisms to *correct* nature's deviances, that is to say diseases, is the great conceptual revolution of the *biotechnological way* to health. We could say that this way is an ecological approach to disease.

Various examples can be reported in surgery. Hemostasis can be obtained through electro-coagulation or other physical, very advanced and efficient methods, or through high local concentrations of coagulation factors, concentrations not present in nature, which highly accelerate the natural coagulation phenomena and produce immediate hemostasis. Through tissue engineering we can have completely or largely *natural* prosthetic substitutions, versus inert or not perfectly integrated prosthesis.

This is the philosophy of the biotechnological approach in medicine and surgery: to consider its great value in leading and letting us be led by nature itself, by vital phenomena in order to correct its own deviances, which in medicine are diseases and their negative consequences.

In the past century we have spoken about a pathophysiologic approach to surgery, where we have tried to use surgery to correct disease without producing major resection and destruction of soma. This philosophic statement was the longing of the surgeon who was nearer nature and less proud of the deep and physical upset he produced, and entitled to do so only by the higher interest in saving life.

To know the phenomenon of *life* in its deepest mechanisms and to find in it the help needed to change deviated nature into the natural and harmonic nature of wellness is the fundamental point of the union between surgeon and biotechnologist. The way we have traced cannot yet be taken in its widest development and moreover it is more backward than the technologic way, especially in surgery.

Surgery has become an exercise requiring very high technologic expertise. The situation of the surgeon today is similar to that of a fighter pilot who has gone from being a bold commander of a jolt vehicle to becoming an aeronautical engineer surrounded by a huge array of equipment in the air cockpit who to fly his craft has to

control the different monitors and not simply look through the windows.

The surgeon, who has always been a technologist because he has always used instruments, even simple ones such as the scalpel, and physical actions such as the fire, has now become the user of numerous technologies present in the operating room and for whom even viewing the surgical field with his own eyes can be limiting, compared with the view offered by systems of navigation and magnification.

Now the biotechnology revolution has entered the operating room and the surgeon has to know, recognize and master it because he is at the summit of his mission: to cure the patient lying on the operating table. Or better still he has to be the fusion point to accomplish his mission. Once again he has to evolve, transform and surpass himself.

The first step in doing so is to believe in this philosophy, to learn the language of biotechnology and to speak with biotechnology researchers. Only after a confrontation and after understanding each other will they be able to use, rapidly and exhaustively the biotechnological discoveries which may risk going unused and losing the objective of the research: human welfare.

This is why the surgeon needs to know biotechnologies, those he already uses in his hospital department every day and those from which he can receive a great help in the future. Only in this way the benefits of biotechnologies will reach the patient early and in the best way. To do this can be expensive or more expensive than a traditional technological approach, but the surgeon must be convinced that biotechnologies and the integration between biotechnology and technology are the way ahead for the future and that they have the added value which at present justifies all financial efforts.

1.1.1 Genomics (fundamentals)

Genomics is the branch of molecular biology which deals with the study of the genome. In particular, it deals with the structure, sequence, content, function and evolution of the genome.

The term genome refers to all the genetic material (DNA or RNA) of an organism needed for survival and replication. Genomics was born in the 1980s when Fred Sanger sequenced the entire genome of a virus and a mitochondrion. In 1986 a public sequencing project began under the name of Human Genome Project with the aim of sequencing the entire human genome, but it was beaten by Celera Genomics (private company) which presented its results a year earlier (2000) than the public project.

The goals of genomics therefore includes the establishment of comprehensive genetic and physical maps of the DNA of living organisms with its complete sequencing.

A very important branch of genomics is transcriptomics, which deals with the expression of genes (mRNA) of a whole organism or a particular organ, tissue or cell at a particular point in the development of the organism or under particular environ-

mental conditions, making major use of microarrays.

A DNA microarray, also called gene chip, DNA chip, or biochip, consists of a collection of microscopic DNA probes attached to a solid surface like glass, plastic, or silicon chip forming an array. These are used to examine the expression profile of a gene or to identify the presence of a gene or a short sequence in a mixture of many genes.

The segment of DNA linked to a solid support is known as a probe and thousands of probes are used simultaneously in an array. This technology arose out of a simpler technique known as Southern blotting, where fragments of DNA attached to a substrate are tested by gene probes with known sequences. The measurement of gene expression using microarrays has substantial interest in the field of basic research and in medical diagnostics, especially for genetic diseases where the genetic expression of normal cells is compared with cells affected by the disease in examination.

Recent developments in gene cloning and sequencing have made possible the identification of genetic causes of many diseases caused by mutations such as triplets (Friedreich's ataxia, X-fragile, myotonic dystrophy) and punctiform (sickle cell, Apert's syndrome) amplification.

1.1.2 Proteomics (fundamentals)

Proteomics is the science which studies the cellular proteome. The proteome is the set of all proteins expressed in a cell, including all isoforms and any possible post-translational modifications. The proteome changes over time, varies in response to external factors and is different between different cell types of the same organism.

Proteomics can be divided into three strands of research:

- systematic proteomics: identifies and characterizes all proteins;
- differential proteomics: studies the differential expression of proteins in different cells of the same organism and in different moments in the life of a single cell;
- functional proteomics: includes the study of interactions between proteins (interactomics), the study of interactions between a protein and its substrates (metabolomics) and the study of specific functions of proteins.

Proteomics is more complex than genomics because the genetic makeup is always constant in all cells of the same body and while about 30,000 genes in the human genome have been identified there are more than 100,000 proteins in human cells.

The study of proteins is performed mainly through biochemical assays such as: Western blot, electrophoresis (2D, SDS-PAGE), ELISA assay, immunoaffinity chromatography, microarray etc.

There is a large number of practical applications of proteomics: pharmaceutical, basic research, identification of new tumor markers, identification of a protein involved in a disease, study of its conformation and function to develop a specific drug (i.e. inhibitor) etc.

1.1.3 Cell Therapy

Cell therapy refers to the introduction of cells, of various origin, in a tissue for the treatment of a disease. Cells can have various origins: autologous (from the same patient), allogeneic (donor) or xenogeneic (from different species) and can be stem or mature cell.

Stem cell treatments are a very important line of research because these particular cells can be differentiated in all mature and functional cells. Stem cells come from two sources: adult or embryo. In clinical practice stem cell therapy is represented by bone marrow transplantation; patients with leukemia or lymphoma are treated with chemotherapy which destroys their aberrant hematopoietic stem cells and is replaced with the bone marrow of a healthy donor or, more recently, with umbilical cord blood stem cells.

In the field of liver disease, transplantation of adult liver cells is a treatment which has been used for several years and which has been proven safe for the patient, especially in pediatric cases, able to improve liver function and delay the need for liver transplantation. Since 1992 transplants have been performed, and many adult human hepatocytes have been implanted in patients with acute or chronic liver failure in an attempt to correct metabolic defects or support liver function until transplantation. In most cases, hepatocytes are isolated from the liver of the patient or non-transplantable organs and injected in the portal vein to enable engraftment in the liver. In other studies, hepatocytes have been infused in the splenic artery, using the spleen as a subsidiary organ to perform liver functions.

For the reconstruction of the epidermis, many substitutes have been developed to permanently cover an injury, including epidermal sheets derived from keratinocytes isolated from adult tissue. The use of autologous epidermal cells for the treatment of skin lesions involves a clear benefit to the patient. However, the use of cell sheets has limits, such as the preparation process, which takes a long time, the low engraftment, which prolongs the period of immobilization required for healing of the patient and costs. The characteristics of fetal cells make them a promising alternative to adult cells to treat skin lesions, as they significantly accelerate the process of wound healing and have a lower expression of histocompatibility antigens on their surface, and thus a reduced immunogenicity compared with mature counterparts.

Adult stem cells can differentiate into a limited number of cellular phenotypes, in general, the obtaining phenotypes are closely correlated with the embryonic tissue source. For this reason they are called multipotent stem cell. Only the embryonic stem cell can differentiate into all cell types (totipotent stem cell) but their use has been much debated. With current technology the creation of a human embryonic stem cell line requires the destruction of a human embryo. Stem cell debates have motivated and reinvigorated the pro-life movement, whose members are concerned with the rights and status of the embryo as an early-aged human life. They believe that embryonic stem cell research instrumentalizes and violates the sanctity of life and is tantamount to murder.

A portion of stem cell researchers use embryos created but not used in in vitro

fertility treatments to derive new stem cell lines. Most of these embryos are destined for destruction, or stored for long periods of time.

Medical researchers widely admit that stem cell research has the potential to dramatically alter approaches to understanding and treating diseases, and to alleviate suffering. In the future, most medical researchers anticipate being able to use technologies derived from stem cell research to treat a variety of diseases and impairments.

1.1.4
Metabolomics: Potential Applications to Surgical Diseases
F. Tarantini, M. Giannelli, D. Boni

The term *metabolomics*, also sometime referred to as metabonomics, was coined in analogy with transcriptomics and proteomics, to indicate the study of global metabolite profiles of a living system. Like the proteome, the *metabolome* is a dynamic entity, changing from second to second. Thousands of small molecules contribute to the construction of the metabolome, which cannot be defined in its totality by any single method of analysis. Age, sex, body composition, genetics, as well as underlying diseases influence the metabolic profile of body fluids. Exogenous factors, such as the large bowel microflora, diet and drugs, are also potential confounders in the study of metabolomics. Detection methods currently employed to identify and quantify metabolites in a biological sample are gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) [1].

The medical challenge which lies ahead is to use the metabolic fingerprint of urine or plasma to identify disease-related modifications and extrapolate the pathophysiologic processes that have produced such a profile. To achieve this aim, complex statistical methods of analysis and pattern-recognition programs have been developed to handle the plethora of data that are generated when healthy and diseased samples are compared.

Metabolomics has been used successfully in toxicology, plant physiology and biomedical research. However, still very few papers have reported the use of metabolomics in humans, for diagnostic purposes.

Metabolomics was applied to patients with renal transplantation [2, 3], with the assumption that a change of metabolite levels in urine could represent an early, specific marker of altered kidney function, allowing early detection of acute cellular allograft rejection. The study demonstrated an excellent association between subclinical allograft rejection and the appearance of specific urinary metabolite profiles.

The most intuitive field in which to envision a possible future clinical application of this method is oncology, given the profoundly altered metabolic profiles displayed by cancerous cells. In this setting, the high sensitivity and specificity of MS or NMR in the detection of metabolites may represent a great advantage for early diagnosis, which is still difficult to obtain for many types of cancer. However, few studies have explored the applicability of metabolomics for tumor detection.

In a study of 85 women with breast cancer, increased RNA metabolism, which is a feature of cancerous mammary cells, allowed for the identification of a specific pattern of urinary excreted nucleosides, able to predict the presence of the disease [4]. Another example is colorectal cancer (CRC), where global metabolic profiling of biopsied colorectal tumors and their matched normal mucosae led to the identification of 31 potential markers of cancer. The metabolites were associated with pathways expected to be perturbed in CRC, such as elevated tissue hypoxia, glycolysis, nucleotide biosynthesis, lipid metabolism and inflammation. Interestingly, colon and rectal tumors displayed different metabolic profiles [5]. A subset of specific urinary nucleosides, identified using a metabolomic approach, correlated with diagnosis of liver cancer better than the traditional tumor marker alpha-fetoprotein, and discriminated between cancer, hepatocirrhosis and hepatitis, reducing the false-positive rate [6]. Lastly, as the metabolic profile of tumors may differ non only between benign and malignant lesions, but also based on pathologic features of cancerous tissues, such as nodal involvement and vascular invasion, the use of metabolomics may guide, in the near future, to a more accurate estimate of disease progression and prognosis [7].

Another very promising field for the application of metabolomics is ischemic diseases. In fact, like tumorigenesis, ischemia profoundly alters tissue metabolism [8]. One attempt to exploit the ischemic metabolic modification of a tissue for diagnostic purposes was carried out in 31 patients with suspected effort angina without previous myocardial infarction. Serum obtained just prior to a diagnostic stress test predicted exercise-inducible ischemia, with lactate, glucose, modified lipids and long-chain amino acids being the main contributors to the discrimination between healthy and diseased samples [9].

Of surgical interest, ischemic bowel disease is an ideal candidate for early diagnosis by metabolomics, which may be able to detect the initial modifications of tissue metabolic pathways induced by hypoxia-anoxia and the change in large bowel microflora secondary to the ischemic insult. An observational study is currently ongoing at the University of Florence to explore this issue.

Metabolomics is a rapidly growing technical approach that has the potential to contribute significantly to clinical practice, complementing more conventional methods of analysis. However, although preliminary results are very encouraging, many obstacles still exist to the widespread use of this technique as a general, diagnostic tool, such as the lack of standardization of different methods of analysis, management of a massive volume of data generated from the screening, implementation of quality control programs. The next few years will tell us if metabolomics will hold its promise to become a complementary/alternative diagnostic device in many medical and surgical fields.

1.2 The Role of the Surgeon in Translational Research

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Translational research is a bidirectional process that creates a strong link between the biology of diseases and clinical interventions. Its complex role is to establish evidence-based medicine and ensure appropriate treatments [10]. Multidisciplinary teams are involved in translational research programs and also surgeons are called to make evidence-based treatment decisions and monitor clinical outcomes. The *bench-to-bedside* approach is the basic concept of translational research and underlines how the therapeutic solutions have to be explored in basic laboratory research and finally tested in randomized clinical trials [11, 12]. Several clinical fields, from cancer to transplantation, have seen surgeons playing a privileged position as research investigator. The participation of surgeons in translational research has been well represented in the past, especially in hormonal therapy for cancer, the field of cardiopulmonary and open cardiac surgery, organ transplantation and organ culture, and in the new biological acquisitions concerning tumor angiogenesis. In these scientific areas, we have to mention Charles Huggins, Gibbon and Lillehei, Joseph Murray and Thomas Starzl, Alexis Carrel and Judah Folkman as unusual examples of the surgeon-scientific vocation [11]. Several scientific-based clinical innovations have been pioneered by surgical scientists trained in the basic sciences, probably due to the unique role of surgeons as directly working with diseased tissues or organs and the clinical context. The translation of molecular knowledge with everyday practice has radically redefined the role of surgery itself. Unfortunately, the increasing complexity inherent in clinical surgical care and basic science methodology has modified the devotion of surgeons to apply basic clinical skills to their research efforts and surgeons have become stagnated with a marginal role in the acquisition of medical knowledge [12]. Clinical behavior based exclusively on macroscopic evidence with a codification of repetitive technical practice is not a scientific method of working. For this reasons, the surgeon must acquire an appropriate and modern mindset in scientific knowledge because his actual role in translational research is marginal. We believe that surgeons might be really effective in translational research if they can demonstrate a greater propensity to learning molecular aspects and more devotion to practical translational projects. The proper arena for the scientific surgeon is the clinical setting and the basic research laboratory because he has the privilege of working with his hands and his mind [12]. The surgeon-scientist has the duty of understanding the essence of clinical observations by asking himself the key *Why* questions instead of stating *We see this*.

1.3 The Role of Biotechnology in Oncologic Surgery

Radical surgery remains the treatment offering the best hope of cure in most patients with solid cancers. However, in the past 20 years, despite technical improvements and despite the reduction of morbidity/mortality after surgery, the results in terms of overall survival have not changed significantly. This situation indicates that cancer surgery (with or without conventional treatments) has reached its intrinsic efficacy limit, at least according to the current information. Advances in early detection, in the identification of subjects at the high risk of developing cancer in hereditary-familial status, and in patient selection may improve the therapeutic efficacy of cancer surgery.

In this context, recent advances in molecular medicine could help to improve cancer surgery results in two main ways [13]:

- developing diagnostic and/or prognostic systems able to identify individuals at risk of developing new cancers (prophylactic surgery), to anticipate early diagnosis (both of primary tumors and of disease relapse), to identify subjects at high risk of developing heredity based cancers, and to select patients needing adjuvant therapies (carriers of minimal residual disease [MRD] after apparently radical surgery) or having the best chance of responding to neoadjuvant treatments (drug and/or radio resistance);
- developing drugs and/or molecularly targeted treatment strategies (highly tumor-specific and therefore highly effective) to eradicate MRD after apparently radical surgery (adjuvant therapy) or to make advanced cancers inoperable (neoadjuvant therapy).

The main types of biotechnology advancement which can take place alongside surgery in the management of cancer patient are:

- the identification of cancer-specific markers which can be used both for the development of new and more effective anticancer therapies and for the identification of cancers sensitive to medical pre- and/or post-surgery treatment and of people at risk of developing a primary cancer (genetic risk, family-hereditary cases) or a recurrent disease (identification of MRD);
- the clinical implementation of new biotechnologies called “high-throughput” for genomics (gene microarrays) [14] and proteomics (e.g. mass spectrometry, protein microarrays) [15] studies, which allow the expression of hundreds/thousands of genes/proteins in biological samples of interest (tumor, healthy tissue, peripheral blood) to be evaluated in a single experiment The molecular profile is highly likely to be much more reliable than the currently used individual molecular markers, because cancer has a multifactorial nature.

Despite the increasing number of drugs proposed for cancer chemoprevention (celecoxib, nitroaspirin, tamoxifen, curcumin, flavonoids, etc.), prophylactic surgery remains the most useful system to prevent some solid cancers (e.g. colon, breast, ovary, thyroid cancer) in populations at risk. However, as the nature of surgery is invasive and mutilating, highly effective predictive-prognostic factors are desirable

to suggest the operation only to people at high risk, compensating the cost (in terms of mobility/mortality and psychological consequences) of prophylactic surgery with a certain benefit.

Until a decade ago familiarity was the only risk factor of developing breast and ovarian cancer in the proband's relatives. The identification of inactivating mutations in two genes coding for DNA repairing proteins (BRCA1 and BRCA2) has pioneered the identification of one of the molecular mechanisms facilitating the transmission of the risk of developing breast and ovarian cancer [16] through germinal tissue. In the general population the frequency of BRCA1/BRCA2 mutation carriers is 0.1-0.2% and their risk of developing breast or ovarian cancer within 70 years is 60-85% or 15-65% respectively. Currently, most authors suggest making genetic tests to find mutations in BRAC1/BRCA2 in women belonging to recognized families affected by hereditary cancer: this allows these individuals at high risk to be put in a screening program for the early detection of breast cancer/ovarian cancer (secondary prevention).

The histologically radical excision of a solid cancer in early stage (stages I and II of the TNM classification) is still the best guarantee of success, as evidenced by the positive results obtained through screening campaigns for breast, cervical cancer and melanoma. The diffusion of screening of a deep organ cancer (e.g. gastrointestinal tract, lung) is thwarted since these can only be investigated by relatively complex and expensive investigations (e.g. colonoscopy, CT). Recent developments in biotechnology (determination of molecular carcinogenesis phases, development of proteomics platforms) suggest that in the near future a simple blood or stool test will be able to identify subjects with a malignancy. For example some authors have shown that the patient's serum protein profile differs significantly from that of a healthy subject [17]. This difference is almost constant in the observed cases, even in the early stages of disease, which makes the test of both scientific and clinical interest (high reliability).

This is a huge step forward from the so-called cancer markers currently available, which can be used only in follow-up if they have increased before treatment, and usually reach significant levels only in advanced cases.

In addition to TNM stage, in recent decades several prognostic factors for the stratification of cancer patients in risk classes have been described. Most of these prognostic markers are represented by individual genes/protein involved in the molecular mechanisms of the biological aggressiveness of cancer, such as p53, p27, p21, Ras, survivin, BCI-2, HER-2Ineu, l3-catenin [13, 18, 19]. However, none of these parameters is characterized by a predictive power and accuracy which would allow their use as binders in each case. On the other hand, the nature of polygenic/multifactorial cancer gives reason for the difficulty of finding/applying a single marker capable of separating patients into two ideal prognostic groups (low versus high risk).

In this sense, the recent implementation of relatively easy to use high-throughput biotechnologies could revolutionize the approach to prognostic factor search. There are now several reports from researchers who were able to identify within the categories TNM subgroups with a different risk by shifting some patients from a lower to

a higher level (thus making them fall in the group of subjects to be treated with adjuvants) and vice versa (sparing them the unnecessary toxicity of treatment) [20, 21].

MRD is a key target for improving surgery results. Currently the task of *sterilizing* any MRD missed by surgical resection of the primary cancer and of metastases is assigned to adjuvant treatments. However it has been estimated that about three quarters of patients undergoing an adjuvant treatment do not need it as they are not MRD bearers, and that the percentage between 20 and 30% of patients who currently are not considered candidates for additional treatments could benefit from them. The fundamental limitation of the current system is the inability to clearly identify patients with MRD.

An example of biotechnological application in surgical oncology is the nodal staging at the molecular level. The histopathological finding of metastases in the sentinel nodes of patients with breast cancer and melanoma is currently an indication for radical lymphadenectomy. However, a variable percentage (10-30%) of micro metastases is not identified through histopathological techniques. The ultramicroscopic metastases (single cells or aggregates) can be detected through biotechnological techniques such as polymerase chain reaction (PCR) and its quantitative version [22]. As regards melanoma, patients with positive PCR have a significantly worse prognosis than those with negative histology and PCR [23].

If these results were confirmed in other solid cancers, the use of biotechnology for the identification of MRD in sentinel lymph nodes may become the standard method for deciding whether or not to submit the patient to radical lymphadenectomy. Moreover, recent technological advances such as the implementation of protocols that allow the automated execution of ultrafast PCR (30 min) may encourage the research of *molecular* metastases during surgery: this would be an advantage for both superficial and deep cancer. In fact, the research of metastases in sentinel lymph nodes during surgery could be used as an ultrasensitive method to guide the surgeon in deciding which type of lymph drainage to perform.

Distant metastases are another aspect in the MRD field. Circulating tumor cells (CTC) can be detected in the patient's peripheral blood usually by means of PCR (standard or quantitative) directed to the amplification of tissue-specific genes (e.g. tyrosinase [melanoma], epithelial antigens [cancer]) not expressed by nucleated cells normally present in blood (white cells). Some authors believe that CTC identification is a promising method for identifying patients who, despite the indications of routine pre- and post-surgery negative staging investigations [24], developed distant metastases. Preoperatively, CTC positivity could lead the choice of surgical timing, by opting for a neoadjuvant treatment, while postoperatively the same positivity could strengthen the indication for an adjuvant systemic chemotherapy.

Molecular medicine has a huge possibility of improving the therapeutic index. As regards more frequently observed cancers, such as in prognostic factors, numerous molecular cancer predictive markers have been reported in the literature, such as thymidylate synthase, Bcl-2, Bax, EGFR, and VEGF. Unfortunately none of the predictors described so far have the requirements (accuracy, predictive power) necessary to select patients proposed for a neoadjuvant therapy, at least when considered individually. However, even in this case, the first encouraging reports on the use of high-

throughput techniques for identifying gene/protein patterns correlating with drug-or radio-sensitivity have been already reported [25, 26].

Although we are still at the dawning of pharmacogenomics/proteomics, the huge expectation for this molecular approach to the problem of drug-radio-resistance/sensitivity finds its firm foundation in the complex nature of these phenomena involving several metabolic pathways such as apoptosis, cell cycle, drug metabolism, and oxide reductive cellular state.

As illustrated in the above data, in the near future, the surgeon-oncologist is likely to obtain a great deal of increasingly large knowledge on biotechnology in order to properly interpret the information on the cancer to be treated which molecular medicine will provide. In fact, recent developments in biotechnology are opening the way for a new approach to cancer patients through the development of more effective drugs (the most active and/or less toxic) and the personalization of treatment. This pressing scientific revolution involves all oncology medical specialties, including surgery.

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New Approach to Diagnosis and Prognosis 2

P. Bechi

2.1 Secondary Peritoneal Carcinomatosis and Biotechnology: a New Approach to Diagnosis and Therapy

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2.1.1 Introduction

Peritoneal carcinomatosis is the spreading of malignant cells into the peritoneal cavity. The peritoneal dissemination of cancer cells is the deposition of malignant cells onto parietal or visceral peritoneal surfaces, associated or less with the accumulation of malignant ascites. The ascites is rich in growth factors, bioactive lipids, extracellular matrix (ECM) components, inflammatory mediators, and proteolytic enzymes, creating a neoplastic microenvironment that fosters further metastatic spread. Primary peritoneal carcinomatosis is rare, whereas a secondary peritoneal carcinomatosis of ovarian (60% of ovarian cancer), gastric (40%) and colorectal cancer (15%) is frequently observed.

2.1.2 Pathophysiology

Pathophysiology and molecular mechanisms underlying the formation of peritoneal carcinomatosis are incompletely understood. Several theories have been proposed

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to explain the peritoneal carcinomatosis (tumor rupture [1], secretion theory, lymphatic theory [2, 3]) and at present molecular research is trying to identify pathophysiologic mechanisms and decrease the time to diagnosis. The development of peritoneal carcinomatosis involves several well-defined steps: detachment of cancer cells from the primary tumor, peritoneal transport, mesothelial adhesion and invasion of submesothelial layers.

Kokenyesi et al. have reported that the expression of the cell–cell adhesion molecule E-cadherin is inversely correlated with the invasive phenotype [4]. Since, the invasive phenotype on type I collagen is mostly lacking of E-cadherin expression, and E-cadherin expression in these invasive cells is regulated at the transcriptional level, immunohistochemical studies have shown that primary ovarian carcinomas have increased levels of E-cadherin [5, 6], whereas ascites-derived ovarian carcinoma cells have diminished levels of E-cadherin mRNA [7, 8].

After detaching from the primary tumor, cellular flow is directed towards the pelvis and from the pelvis, along the right paracolic gutter, towards the subdiaphragmatic space according to the force and the physiologic interaction described by Meyers [9] and to spontaneous cell mobility. In fact, growth factors and their receptors are products of protooncogenes, which when expressed pathologically, activate both the actin microfilaments-system and gene expression, resulting in increased motile activity and cell proliferation [10].

Intraperitoneal dissemination needs the ability of neoplastic cells to interact with a variety of matrix components, such as fibronectin and vitronectin, deposited on the peritoneal surface and basement membrane, and laminin, which is a major component of basement membrane. Many cell adhesion molecules (CAMs) play a role, such as ICAM-1, VCAM-1, PCAM-1, E-selectine, mesothelin, CA125 and MUC16. In particular, mesothelial cells express a set of CAMs. These molecules, for instance, are important in the inflammatory response; in fact, the transmigration of leukocytes into inflamed tissues requires a chemotactic stimulus and the engagement of platelet-endothelial cell adhesion molecule-1 (PECAM-1) [11]. Furthermore, the mesothelial cell intercellular adhesion molecule-1 (ICAM-1) has recently shown to be important for the tumor cell adherence to the peritoneum, while CD43 is expressed by a variety of carcinoma cell lines and plays a role in the tumor cell-peritoneal adhesion probably by interactions with its putative ligand ICAM-1 [12]. Ovarian carcinoma cells express several adhesion molecules, including CD44 and other integrins, but only an anti-CD44 antibody has been shown to be capable of inhibiting mesothelial binding. The results of the study by Cannistra et al. [13] suggest that CD44H may be an important mediator of ovarian cancer cell implantation and that decreased CD44H expression may be associated with release of cells into the peritoneal space during ascites formation. Cultured mesothelial cells are very responsive to the proinflammatory cytokines, IL-1 etc. Moreover peritoneal neoplastic growth is stimulated by the post-operative increase of the plasmatic concentration of VEGF [14].

Sawada et al. [15] have shown that c-Met (a receptor tyrosine kinase which plays an important role in tumor growth) overexpression is a prognostic factor in ovarian cancer.

Loose tumor cells might enter the submesothelial tissue at areas of peritoneal discontinuity or alternatively tumor cells can induce apoptosis of mesothelial cells. Invasion of the submesothelial tissue is accompanied by adhesion of integrins to the extracellular matrix and degradation by proteases (MMP-7, MMP-9, WT1) [16, 17].

Several studies about genetic profile of colorectal neoplastic cells have shown different genetic expression, so a classification of patients based on metastatic site is possible: predominantly peritoneal spreading (TIMP-2, IGF-1, and overexpression), hepatic (TIMP-1) or both solutions (CDH17 and MTDH) [18, 19].

2.1.3 Future View to Diagnosis and Therapy

Pathophysiology, molecular mechanisms and the role of micro environmental factors in the development and progression of peritoneal carcinomatosis are not well understood.

The lack of reliable biomarkers for widespread early detection combined with relatively nonspecific symptoms lead to a delay in the diagnosis in many new patients. Currently, routine cytology to detect free peritoneal cancer cells is associated with low sensitivity.

In recent years several studies have shown the interaction between endothelial and neoplastic cells about hematologic metastasis mechanisms, in particular, a set of genes which mediate breast cancer metastasis to lung and is clinically correlated with the development of lung metastasis when expressed in primary breast cancers [20]. Probably, in the future, this knowledge will be applied to peritoneal tumor spread.

One study performed on 30 colorectal cancer patients seems to suggest that presurgical IL-2 immunotherapy may counteract surgery-induced stimulation of angiogenesis, by either opposing the decline in blood levels of the antiangiogenic cytokine IL-12 or reducing the increase of the angiogenic factor VEGF [21]. This may determine an increase in postoperative complications [22].

According to the study by Cannistra et al. [13], strategies which interfere with CD44H function may result in decreased intraabdominal spread of a highly lethal neoplasm. In addition, c-Met should be explored further as a therapeutic target in ovarian cancer, since targeting c-Met in vivo inhibits peritoneal dissemination [15].

With regard to treatment, HER2/neu overexpression is observed in 10%-30% of ovarian cancer cases and associated with poor prognosis. Moreover, this gene amplification has also been observed in bladder, breast, colon, stomach, and lung cancers. Helguera et al have shown that treatments with AbFPs can effectively generate high levels of protection against peritoneal tumors expressing HER2/neu, which may be relevant in patients with primary or metastatic peritoneal carcinomatosis which may be observed in ovarian, colon, stomach, bladder, lung, and breast cancers [23].

The intraperitoneal administration of heparin appears to be correlated with a decrease in ICAM-1 expression and cellular adhesion [22]. The anticancer activity of heparins depends more on the inhibition of metastasis formation than on the

effects on primary tumor growth. These effects are probably related to both coagulation and non-coagulation dependent factors [24].

In preclinical models, the use of monoclonal antibodies, anti-integrin, antiadhesion molecule (L1, JAM-C) has shown a decrease in tumor growth and adhesion.

The results of several studies and some ideas that have arisen in the field of endothelial cell and tumor interaction and hematogenous metastasis formation will probably be applied in the near future to the study of peritoneal dissemination. Moreover, the identification of metastasis-specific genes activated early in the primary tumor might make it possible to predict the tumor's behavior.

Among future developments it remains to be elucidated whether some molecules or antibodies will be used in diagnostic and therapeutic activity.

In this respect we are performing a study involving 75 enrolled patients (affected with gastric, colorectal or ovarian tumor). The primary endpoint is the analysis of some circulating molecules targeted to an early diagnosis of peritoneal carcinomatosis. A secondary endpoint is the evaluation of the molecular difference between primary tumors with high metastatic capacity and those with low metastatic capacity.

2.2 Pre-endothelial Cells and Prognosis of Intestinal Adenocarcinoma

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2.2.1 Introduction

It is well known that the generation of new and functional vessels is one of the crucial hallmarks of cancer progression [25]. A number of ongoing studies have been published concerning the relationship between angiogenesis and colon cancer (prognosis, therapeutic targets, angiogenesis and advanced and metastatic disease). Until recently, it was generally accepted that in adults blood vessel formation is determined exclusively by completely differentiated endothelial cells (ECs). Recent studies have shown, however, that circulating bone marrow (BM) derived endothelial progenitor cells (EPCs) contribute to neovascularization and participate in new vessel formation, in a process called *postnatal vasculogenesis* [26]. Moreover, recent studies using different animal models of cancer have suggested the importance of BM-EPCs in tumor vascularization and growth [27, 28], and some evidence suggests that circulating EPCs are increased in cancer patients. EPCs are present in the peripheral blood, their levels are increased in response to certain signals/cytokines, and they dwell into the neovascular bed of malignant tissues. Therefore, on the basis of these observations, EPCs have attractive potential diagnostic and therapeutic applications for malignant diseases and the measurement of circulating EPC (C-EPC) has potential applications in colorectal cancer patients both in prognosis and therapy

2.2.2
Endothelial Progenitor Cells and Post-natal Vasculogenesis

Asahara et al. were able to show for the first time that C-EPCs exist and are recruited for tumor neovascularization [29]. It was therefore shown that new blood vessel formation in adults can also be provided by postnatal vasculogenesis. Extensive studies during the last 10 years have led to the identification and characterization of the peripheral blood (C-EPCs) and BM-EPCs as the sources of EPCs [30, 31], able to migrate through the tissue and to organize in tubules that eventually mature into vessels. These cells show the ability to repair the endothelium and to participate in the angiogenesis process, so it is conceivable that in any given moment the level of C-EPCs reflects the endothelial endogenous regenerative capacity of a patient. Thus, unlike circulating mature endothelial cells (CECs), which derive from mature endothelium and are considered a marker of vascular damage, EPCs are believed to be vasculoprotective and causally related to endothelial integrity and angiogenesis [32].

2.2.3
EPCs, Tumor Angiogenesis and Metastasis

Primary tumors, as well as metastatic neoplasms, need to undergo the *angiogenic switch* in order to grow beyond the microscopic dimension. The angiogenic switch is induced by soluble factors produced by tumor cells as well as other kinds of mesenchymal cells, nontumor cells (fibroblasts, macrophages) which constitute the tumor microenvironment. This process is a key step in the relapse from the tumor dormancy which allows the metastasis outgrowth. Experimental tumor models have shown a tumor-type dependent involvement of EPCs in tumor vascularization [33-35]. However, conflicting results exist. In some recent studies it has been shown that BM-EPCs contribute to the generation of peri-endothelial cells rather than endothelial cells and to new vessel formation [35, 36]. In mice transplantation studies, donor BM-derived EPCs integrated into newly formed blood vessels, sometimes by as much as 50% [35, 37], whereas other studies have reported significantly lower levels [38]. Recent published in vivo data suggest that small amounts of EPCs (12%) are sufficient to promote the vascularization of metastatic lesions [25, 29]. These conflicting results are mainly due to the still not exactly standardized methods for EPC phenotypic characterization via detection of cell surface markers (see Section 2.4) and to the non consideration of their functional interaction with other cell types able to contribute to the angiogenesis process [35, 39]. The involvement of EPCs in tumor angiogenesis has also been demonstrated in clinical studies. Increased EPC values have been detected in the circulation of cancer patients [40]. In one clinical study, tumors from six patients who developed cancers after BM transplantation were found to contain an average of 5% of EPCs from the donor, most likely generated by C-EPCs [41].

The contribution of EPCs to metastasis vascularization and to metastasis support

is largely confirmed [42-44]. Kaplan et al. [44] have indeed found that VEGFR-1 positive hematopoietic cells (HPCs) derived from bone marrow act as a guide to drive circulating tumor cells to their metastatic sites. In these, HPCs train the soil for the subsequent arrival of cancer cells in the so called *metastatic niche*. In this context, EPCs participate in the construction of the metastatic niche arriving at this site concomitantly with tumor cells and hence, before vascularization occurs.

2.2.4 EPCs as a Potential Biomarker for Tumor Angiogenesis

Colon carcinoma is included in the tumors which are angiogenesis-dependent so colon cancer patients are also treated with the bevacizumab, an anti-VEGF antibody. Antiangiogenic treatments for cancer could be improved if reliable surrogate markers of drug biological activity were available. Many preclinical angiogenesis assays rely on the growth-factor-induced generation and quantification of neovessels at sites such as the cornea, skin or dorsal sac. Such surrogates are not adaptable to patients. The measurement of changes in tumor angiogenesis as a means of predicting and/or assessing the efficacy of antiangiogenic therapies has mainly been based on the evaluation of microvessel density in tumor biopsy samples [45]. Another approach is the measurement of circulating levels of angiogenic growth factors, such as VEGF, FGF-2, HGF, IL8 and others. In some types of cancer, these growth factor levels could predict survival. However, no growth factor has so far been validated in predicting the response to antiangiogenic therapies [46]. A promising strategy is the measurement of C-EPCs [47]. However, a lack of consensus about the definition of these precursor cells, using different cell surface markers, provides difficulty in the interpretation of these and other data [47]. These cells are phenotypically characterized by flow cytometry due to the expression of at least 2 hematopoietic stem cell markers ($CD133^+$or $CD34^+$), the endothelial marker VEGF-receptor 2 (VEGFR-2) or CD31. Thus, $CD34^+$ $VEGFR\text{-}2^+$, $CD133^+$ $VEGFR\text{-}2^+$, $CD34^+CD133^+$ $VEGFR\text{-}2^+$, $CD34^+CD31^+$, $CD133^+CD31^+$ and $CD34^+CD133^+CD31^+$ are all theoretically possible EPC phenotypes. In vitro studies have suggested that also $CD34^+/CD45^-$ cells have a higher capacity to acquire an endothelial phenotype, whereas $CD34^+CD133^+$ $VEGFR\text{-}2^+$ have recently been described to be primitive HPCs instead of EPCs [48]. Moreover, it should be noted that C-EPCs share membrane markers with CECs and HPCs and, in some case, with platelets and microparticles [32]. As further summarized in Table 2.1 there are no antigen-specific antibodies which, when used alone, can discriminate between C-EPCs, CEC, platelets and HPCs. For example, CD146 was an endothelial-specific marker, but more recent evidence indicates that it is also expressed by some mesenchymal cells, by a subpopulation of activated lymphocytes and most importantly by EPCs [47, 49]. At present, only CD133 is the sole antigen expressed in C-EPCs and subsequently downregulated in mature CECs, even though in humans CD133 is also expressed by HPCs. It seems that a panel of antibodies for different antigens should be used to

Table 2.1 Phenotypic differences of endothelial progenitor cells, circulating endothelial cells and hematopoietic progenitor cells

Marker	C-EPCs	CECs	HPCs	platelets	EC micro-particles
CD34	++	+/-	++	+/-	+/-
CD133	++	-	++	-	-
CD117	+	-	++	-	-
CD31	+	++	+	++	++
CD45	+/-	-	+	++	-
CD144	++	++	+/-	-	++
CD146	++	++	+/-	-	++
VEGF-R2	++	+	+/-	-	-/+
VWf	+	++	-	+	++

CECs, circulating endothelial cells; *EPCs*, endothelial progenitor cells; *HPCs*, hematopoietic progenitor cells

discriminate both C-EPC and the above mentioned cell population. In an attempt to develop a robust clinically useful FACS protocol to study C-EPCs and/or CEC, it is conceivable to define CECs as cells expressing $CD31^{bright}$ $CD45^{-}$ $CD34^{dim}$ $CD133^{-}$ and C-EPCs as $CD31^{+}$ $CD45^{dim}$ $CD34^{bright}$ $CD133^{+}$ [50].

The clinical relevance C- EPCs in tumor patients is under investigation. Recent studies reported an EPC increase in cancer patients, suggesting that these precursors contribute to postnatal vessel formation [35, 47]. C-EPCs might correlate with invasive breast cancer [51], with lung [52], head and neck cancer patients [53] and with the levels of circulating VEGF in lymphoma patients [54]. Elevated circulating endothelial progenitor marker CD133 mRNA levels have been detected in colon cancer patients and predict malignancy recurrence [55], although larger prospective studies are needed. The number of C-EPCs in chemotherapy-treated patients has also been correlated with survival [56]. The characterization of C-EPCs in relation to the clinical setting represents an attractive surrogate marker for monitoring tumor responses to conventional and to antiangiogenic therapy [57]. It has been demonstrated, indeed, that endostatin reduced C-EPC number along with tumor regression [58]. Moreover, clinical studies have been undertaken in patients treated with antiangiogenic drugs. Particularly encouraging in this regard is a trial (now Phase II) with the anti-VEGF antibody bevacizumab which has demonstrated its ability to reduce the tumor vascular density and the number of EPCs in rectal carcinoma patients [59]. This is the reason why there is an urgent need of clinical trials and standardized methods to characterize C-EPCs in cancer patients, in order to not only assess antiangiogenic therapy, but also to help define the optimal dose range and the establishment of the appropriate tumor response criteria.

2.2.5 Molecular Markers of Tumor Angiogenesis and Antiangiogenic Drug Activity

Molecular markers can certainly improve our understanding of prognosis and the treatment of cancer. This is true also for colorectal cancer (CRC). CRC is a heterogeneous disease resulting from the co-occurrence of oncogenic mutations in a multistep process. Studying molecular classification and molecular correlates can provide clues to the pathogenesis and provide surrogate markers in clinical or research studies [60]. Many attempts are currently being made to obtain a precise molecular fingerprinting of both EC and EPCs, by searching for proteins such as tumor angiogenesis biomarkers. In addition, the possible definition of these new biomarkers will be helpful in clinical situations such as relapse after surgery or chemotherapy. Hence, EPC biomarkers will be useful to monitor either chemotherapeutic and/or antiangiogenic drug activity. In addition to the enumeration of C-EPC through FACS analysis (flow cytometry) of their surface markers, an overexpression of mRNA levels in EPC derived from tumor specimens compared to EPCs of normal tissues has been found [61]. The measurement of cancer-related EC and EPC populations looks promising, but standardized protocols are needed in view of this possible clinical use.

2.2.6 EPC as Cellular Vehicles for Anticancer Therapy

It has also been suggested that EPCs could be used as a vehicle for anticancer therapy. This might be achieved by ex vivo manipulation of EPCs in order to deliver suicide genes, toxins, or antiangiogenic drugs. This novel approach has been applied in mice with promising results [62].

2.2.7 Conclusions and Future Perspectives

Our Institution is carrying out a study on EPCs and colon cancer by means of the collection of blood samples from patients with CRC and investigating each patient's matched normal colon mucosa and CRC tissue, resected from the surgical specimen.

The immunohistochemical evaluation and flow cytometry analysis on tumor and draining lymph nodes specimens is performed by using the following mAb: CEA, MHC class I and II molecules, CD4 and CD8, CD16 and CD56.

Blood samples are taken prior to surgery and follow-up samples are taken after 24, 48, 72 h and 1 week after surgery, during chemotherapy and after 3 months of chemotherapy.

EPCs contribute to tumor angiogenesis and seem to play a crucial role in the guidepost of tumor cells TP to the metastatic niche. However, it is not currently clear what is their relative contribution to post-natal vasculogenesis when compared

to that of in situ angiogenesis (i.e. endothelial sprouting). Moreover, it can be assumed that new antiangiogenic drugs might also have significant side effects on the cardiovascular system. Surrogate markers capable of predicting not only the clinical anticancer benefit, but also the side effects will be needed. Short- and long-term studies of CEC and EPC kinetics, phenotype, activation and viability, both in response to antiangiogenic drugs will become increasingly important in helping to answer these questions.

2.3 Microarray Gene Expression of Pancreatic Carcinoma

D. Pantalone, I. Giotti, V. Ceccherini

2.3.1 Introduction

Pancreatic cancer is still a highly lethal disease if not the most lethal disease of all the solid malignancies [63]. It has been estimated that worldwide, approximately 213,000 people were diagnosed with a pancreatic cancer in 2005 [64]. In Italy, it is the tenth malignancy in women in terms of new diagnoses and the eleventh in men with about 9,000 new cases each year. It is the sixth cause of death from cancer in women and the seventh in men. It is also the second cause of death from gastrointestinal cancer. The high mortality of pancreatic adenocarcinomas can be attributed to the fact that the overwhelming majority of patients present locally advanced or distant metastatic disease, which therefore is inoperable. However, even among patients presenting resectable pancreatic cancers, currently only approximately 15% have the earliest stage cancers (T1, or T2, N0). Those cancers have a better survival outcome, but only 2-3% of all patients presents at an early stage. At present, the detection of pancreatic cancer at an early and therefore potentially curable stage remains the best possibility of improving the patient survival [65].

Unfortunately, the screening tests capable of detecting pancreatic cancer at an early stage are not available. Appropriate cancer screening should lead to the early asymptomatic or unrecognized cancer detection by means of acceptable and inexpensive widely applicable tests.

A better knowledge of genetic modification may be an important step for both cancer diagnosis and treatment and could provide evidence for the identification of early markers. Recent studies demonstrate that a high risk of developing pancreatic cancer can be identified in a patient's family history. An improvement in the identification of a high risk subject for pancreatic cancer will be provided by the identification of genetic alterations that predispose the inherited forms of pancreatic cancer. While it is important to target a high-risk family, this strategy does not facilitate the early detection of sporadic forms of pancreatic cancer.

2.3.2 Microarray

Although the role of some known oncogenes and tumor suppressor genes in the development of human pancreatic adenocarcinoma is nowadays fairly well established, it is quite obvious that, considering the complexity of the genome, the majority of the specific genetic changes involved in the initiation and progression of this disease remain unknown [66]. Genetic alterations influencing the expression and activity of tumor suppressors or oncogenes such as KRAS2, CDKN2A, TP53 and DPC4 have been directly implicated and thoroughly analyzed, for the most part, by conventional methods [67], such as comparative genomic hybridization (CGH) [68-70].

Genome-wide analysis of gene expression by using microarrays has recently received a great deal of attention, particularly due to its higher resolution when compared to that of traditional comparative genomic hybridization [71]. Microarray technology was originally developed to study differential gene expression using complex populations of RNA [72]. Improvements in the methods of the technique now allow the analysis of copy number imbalances and gene amplification [73] and have also been applied to the systematic analysis of expression at the protein level [74], as many of the key principles of this technique are applicable at the RNA, DNA or protein level.

The principle of a microarray experiment is that mRNA from a given cell line or tissue is used to produce a labeled sample, termed the *target*, which is hybridized in parallel to a large number of DNA sequences, immobilized on a solid surface in an ordered array. Tens of thousands of transcript species can be detected and quantified in a single experiment. Currently available microarrays in theory permit the analysis of all genes in the human genome simultaneously and provide a really high resolution which only depends on the number of DNA fragments and their spacing along the genome.

The most frequently used methods can be divided into two groups, depending on the arrayed material: complementary DNA (cDNA) and oligonucleotide microarrays. Probes for cDNA arrays are usually produced by the polymerase chain reaction (PCR), generated from cDNA libraries, and are printed onto glass slides as spots at defined locations (Stanford technology). Using this system, arrays composed by more than 30,000 cDNAs can be fitted onto the surface of a conventional microscope slide. For oligonucleotide arrays, short 20-25mers are synthesized *in situ*, either by photolithography [75] (Affymetrix technology [76]) or by ink-jet technology (developed by Rosetta Inpharmatics [77]).

To prepare the target, mRNA from cells or tissue is extracted, converted to DNA and then labeled with fluorescent dyes, hybridized to the DNA elements on the surface of the array, and detected by phospho-imaging or fluorescence scanning. When using different fluorescent dyes it is possible to label mRNAs from two different cell populations or tissues in different colors that can be then mixed and hybridized to the same array, resulting in competitive binding of the target to the arrayed sequences. However, as the efficiency of incorporation of nucleotides labeled with

different fluorescent dyes during target-sample preparation may not be equal, reciprocal labeling with swapped colors is recommended [78]. After hybridization, the slide is scanned using two different wavelengths, corresponding to the dyes used, and the intensity of the same spot in both channels is compared. This results in a measurement of the ratio of transcript levels for each gene represented on the array.

The data of a microarray experiment typically constitute a long list of measurements of spot intensities and intensity ratios, generated either by pairwise comparison of two samples or by comparing several samples to a common control. The challenge is then to sieve through this huge amount of data to find significant results.

Due to the complexity of the data sets produced by microarray experiments, the quality of the data analysis software is crucial. Many data-analysis devices have been developed by commercial suppliers (such as GeneSpring from SiliconGenetics, http://www.sigenetics.com/), whereas others are available from public sources [79].

2.3.3 Microarray and Pancreatic Cancer

The results produced by a few different research groups show that meaningful data can be obtained by gene expression profiling of pancreatic adenocarcinoma using high-density arrays. However, the published results are not so easily comparable. Only a few genes have been shown to be differentially expressed in more than one study. There are several reasons which could explain the low concordance of these studies. First of all, the type, the histology, and the number of samples used (i.e. established cell lines or pancreatic ductal adenocarcinoma cells) are different. Secondly, different arrays and array technologies may produce different gene expression results. Kuo et al. [80] showed a very poor correlation when two different types of DNA microarray technology were used (cDNA versus Affymetrix oligonucleotide microarrays) but the same cell culture samples. Finally, a gold standard for statistical analysis and data mining has not been established yet. For these reasons the results of expression profiling studies in pancreatic ductal adenocarcinoma are not yet completely comparable [81].

Microarray technology has been proven to be practicable in pancreatic cancer, as many novel candidate genes have come out from these studies. However, individual cancer stages, grading, and TNM classification have not yet been proven to correlate with the expression profiles. In addition, molecular differences between long- and short-term survivors have not been detected. However, the small number of analyzed samples with different series might be responsible at this moment for these disappointing results.

A comparison of the results of eight studies [82-89] which analyzed the genome-wide alterations of the adenocarcinoma of the pancreas shows that a certain amount of genes were differentially expressed in more than one study. The majority of these genes (n=70) were found to be upregulated in pancreatic adenocarcinoma. One gene, S100 calcium-binding protein P, was upregulated in five of the eight studies. Four genes, including annexin A1, integrin beta 4, and laminin gamma 2, were upregulated

in four studies. Nineteen genes were found to be overexpressed in three studies, including lipocalin 2 and urokinase plasminogen activator receptor, two genes that had already been shown to be implicated in the carcinogenesis of pancreatic cancer [90, 91]. In two studies most of the genes – 46 in total – were upregulated. Of the genes that were overexpressed in pancreatic cancer, nine genes, including keratin 7 [92], stratifin [93], and CD55 [94] have been previously implicated in the development of this disease. These results, which have also been identified by other profiling methods, confirm the validity of microarray-based expression profiling. Moreover, 14 of the upregulated genes were known to be implicated in other cancers, but not in pancreatic cancer so far. Among these genes there are such genes as versican (overexpressed in malignant melanomas) [95], insulin-like growth factor-binding protein 3 (overexpressed in non-small cell lung cancer and breast cancer) [96] and carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) (overexpressed in colorectal carcinomas) [97]. These genes might be valuable candidates for genes which could play a role in pancreatic cancer as well. The remaining 13 genes have not previously been implicated in carcinogenesis. Only 16 genes, including PDCD4 and Annexin A8, were found to be downregulated in PDAC in two or three of the eight publications. One gene, nucleosome assembly protein 1-like 1 (NAP1L1), showed divergent results: two studies described an upregulated status of this gene, whereas in another study it was described as downregulated using microdissected material.

2.3.4 Conclusions

Since 2000 our group has been actively involved in scientific research on pancreatic adenocarcinoma. A pilot study showed that some of the typical genetic alterations of pancreatic adenocarcinoma could also be observed when analyzing samples of bile [98].

In the period between October 2002 and December 2009, 44 patients affected by this disease have undergone surgery at our surgical unit. The specimens of pancreatic tissue were employed to establish 4 new neoplastic cell lines (called PP78, PP109, PP117 and PP171), which were used in our molecular biology studies as an alternative to fresh tumor samples. These cell lines were characterized using traditional methods, such as RT-PCR analysis, kariotype analysis, Western Blot analysis, etc.

Our preliminary results showed that, as expected, a large amount of genes were differentially expressed in neoplastic cells and that some of these genes were implied in the same biologic and metabolic pathways, such as the signal transduction or the regulation of immune response, thus suggesting that these processes have some role in the pathogenesis or progression of pancreatic adenocarcinoma.

Although this technique is decidedly complex and needs dedicated technology, it is able to collect an extraordinary amount of information. The value and potential of the method in clinical applications deserve a further effort for the classification and categorization of specific genetic changes involved in the initiation and progression of this disease.

2.4
The Significance of Circulating Tumor Cells as a Prognostic Marker for Colon Cancer: a New Biotechnology

A. Taddei, F. Castiglione, M.N. Ringressi

2.4.1
Introduction

Colorectal cancer (CRC) is the second most common cause of cancer related death in Europe and the United States. Mortality rates for colon cancer has fallen during the past 10-15 years, due to early detection, more effective screening programs and adjuvant and neo-adjuvant chemotherapeutic treatments which have led to a significant increase in tumor response and median survival [99]. Approximately 40-50% of CRC patients who have undergone a supposedly curative resection subsequently develop metastatic disease and die within 5 years [100]. Survival is closely related to the pathologic stage of this disease. There is a dramatic difference in survival rates between patients with early-stage CRC and those with advanced CRC [101].

In recent years, several highly sensitive methods have been developed to detect altered expressions of tumor markers in the blood of patients with different types of malignancies. Detection and quantification of circulating cancer markers in the whole blood sample may prove to be a unique and easy diagnostic tool for determining the prognosis and therapeutic effectiveness of the cancer therapy. Differences in the detection methodology, types and numbers of target genes or antigens, sampling site and time, as well as in demographic or clinicopathologic data of patients included in the studies are present in the literature and are considered potential sources of heterogeneity.

Since the mid-19th century, scientists have described microscopic aggregates of tumor cells in the blood of cancer patients. Known as circulating tumor cells (CTC), they are thought to play a role in the process of metastasis by becoming detached from a solid tumor, entering the bloodstream, and eventually migrating to distant organs to develop secondary tumors. CTC can be detected by two different techniques: cell search system and RT-PCR. Cell search utilizes antibodies that are joined to microscopic iron particles, called ferrofluid. These antibody/ferrofluid combinations are capable of forming a very specific and tight bond with CTCs. These complexes are then drawn out of the blood samples by means of powerful magnets. Further treatment by means of staining and additional biomolecules and/or chemicals enables their positive identification as CTCs. A potentially more sensitive molecular approach for the detection of CTC is polymerase chain reaction after reverse transcription (RT-PCR) in its standard form as quantitative PCR or real-time. This has been applied to various cancers targeted to a variety of markers transcripts which are sited on the cell surface such as low molecular weight cytokeratine (CK) and CEA. The first study concerning this method was published in 1991; later on, many authors reported molecular diagnostic techniques in the lymph nodes and in the blood and bone marrow of patients with cancer. The application of RT-PCR in the regional

lymph nodes and sentinel lymph nodes was described for different neoplasms, including melanoma, colorectal, prostate, breast and lung cancers. Most of these studies compared ICC sensitivity and specificity with RT-PCR and they concluded that the RT-PCR may be a better choice, provided that the markers are sufficiently specific, as carcinoembryonic antigen (CEA) certainly is for CRC.

CEA is a glycoprotein localized in the apical surface of mature enterocytes. The members of the CEA gene family are clustered on chromosome 19q13.2. It consists of 29 genes, 18 of which are expressed. Many functions of CEA have been known in healthy patients, however its role as cell adhesion molecule is the most studied. CEA was first described in 1965 by Gold and Freedman [102], when they identified an antigen that was present in both fetal colon and colon adenocarcinoma but that appeared to be absent from the healthy adult colon. Since the protein was only detected in cancer and in the embryonic tissue only, it was given the name carcinoembryonic antigen. Thirty years after its initial detection in serum, CEA is one of the most widely used tumor markers throughout the world and certainly the most frequently used marker in CRC [103, 104]. Therefore, in CRC, CEA may be the ideal marker on cell surface for CTC evaluation by means of RT-PCR [105, 106]. In this respect carcinoembryonic antigen-related cellular adhesion molecule (CEACAM3), one of its isoforms, has been hypothesized as particularly suitable for CTC detection [107, 108].

2.4.2 Personal Experience

The purpose of our study, which is at a preliminary stage, is to evaluate whether RT-PCR assessment of CEA is a reliable method for evaluating CTC in peripheral blood of CRC patients. So far, only the RT-PCR has been shown to be feasible and at the same time sensitive enough to detect the CRC markers, which are present, although in low quantity, in whole blood samples. Therefore, we have evaluated gene expression of CEACAM 3 as target molecule for the detection and quantification of one of the specific markers by RT-PCR.

Samples of whole peripheral blood from 50 consecutive patients (28 males, 22 females, mean age 71) have been obtained from patients undergoing colon resection for sigmoid cancer. Controls are represented by blood samples from 12 healthy volunteers. Samples have been taken from brachial vein before surgical operation and at the first and fifth postoperative day. None of the CRC patients had undergone preoperative radio- / chemotherapy.

RNA was isolated using 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol, and afterwards stored at -80°C. All RNA samples (200 ng) were reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Biorad, Hercules, CA) according to the manufacturer's protocol. Negative control without RNA was performed. GAPDH was used as endogenous control gene for normalization and was detected using gene-specific primers and probes labeled with reporter day VIC (Applied Bio systems, Foster City, CA). PCR reaction was carried out in triplicate on 96-well plate with 20 µl each well using ox Taman

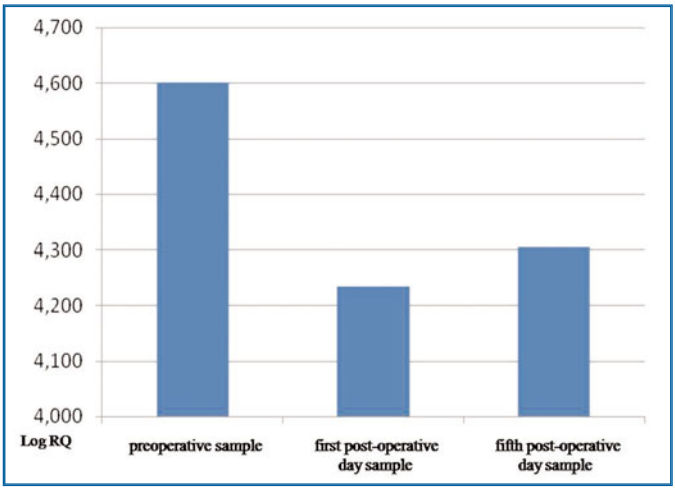

Fig. 2.1 Relative expression of CEACAM3 gene in samples from patients undergoing colon resection

Universal PCR Master Mix. After an incubation for 2 min at 50 °C and 10 min at 95 °C, the reaction continue for 50 cycles at 95 °C for 15 s and 60 °C for 1 min.

One hundred and sixty-two samples of whole peripheral blood from 50 consecutive CRC patients and 12 healthy subjects were analyzed. RNA was successfully extracted from all the samples. CEA was expressed in 49 of the 50 CRC patients; it was not expressed in the control subjects. Moreover, CEACAM3 evaluation shows a higher expression of CEA in the pre-operative samples when compared to postoperative samples (Fig 2.1; $p<0.05$) with no differences between the early and late post-operative samples.

2.4.3 Conclusions

CEA is a particularly relevant marker because it is expressed in a variety of adenocarcinomas such as colon, rectum, pancreas, gastric, breast, etc. The present preliminary study seems to show that CEA is detectable by means of RT-PCR in patients affected with CRC and is not detectable in control subjects. Since, for the above mentioned reasons, CEACAM3 levels can be considered a measure of the CTC number, it seems noteworthy that its levels decrease after surgery. On the clinical ground, therapy could be importantly affected by CTC findings. For example, selection of patients for adjuvant chemotherapy as well as monitoring of response may be a good indication to their assessment. However, the best method of detection, the optimal sampling times and other methodological aspects are still controversial.

In conclusion, evidence stands for a strong prognostic impact of CTC in CRC patients but, at the moment, the reference standard technique for CTC detection has not yet been defined, although RT-PCR, as confirmed by our experience, appears to be the most promising.

2.5 Advanced Diagnostic Applications

G. Cafiero, F. Papale, A. Barbarisi

2.5.1 Introduction

In principle, an electron microscope works like a normal optical microscope and is essentially composed of an electronic source (usually an incandescent filament) and a device that gives strong acceleration to the electron. The beam of electrons accelerated through a capacitor (electrostatic or magnetic) has an impact on the sample, is collected by a probe that turns it into an electronic signal which is then processed by a computer to form the image for visual observation.

The scanning electron microscope (SEM), provides information on appearance, the nature and properties of surfaces and layers of usually solid samples, with an average resolution of 2-5 nanometers.

The most common technique for obtaining images from an SEM is to coat the sample surface with a thin metal film produced by sputtering or vacuum evaporation. This is the fundamental limitation of SEM, since the dehydration and metallization of the sample entails the risk of creating artifacts and the inability to observe samples in their natural condition. In recent years a method called environmental scanning electron microscopy (ESEM) was developed from the SEM, but the sample is hydrated and non-metallic.

The structure of the ESEM is very similar to the SEM, but the sample is not subjected to high vacuum to allow some of the hydration to be maintained. The degree of hydration of the sample can be controlled by varying the pressure and temperature, which allows the study of real-time changes in the structure and status of the sample [106].

2.5.2 Immunogold Labeling

The most common immunohistochemical technique involves the use of two antibodies called primary and secondary. The primary antibody recognizes a specific antigen and binds to it while the secondary antibody recognizes and binds the Fc fragment of the primary immunoglobulin, which is conjugated to a substance which can be viewed with an optical microscope (chromophores or fluorescent) or electron microscope (metallic nanoparticles).

Immunogold labeling (IGL) is the correspondent of histochemical staining in light microscopy, whereby secondary antibodies are conjugated with gold nanoparticles. Gold is used because it is a very ductile and malleable metal which can have nanoparticles with a diameter of about 1.5 nm. The technique of silver enhancement (SE) is designed to increase the diameter of nanospheres through the deposition of

silver ions around the gold in the presence of a reducing agent. With this method the particles can be enlarged up to 100 nm, making them visible even at *relatively* low magnifications which provide an overview of the cell.

2.5.3 Thyroid Lesion

Thyroid cancer is not very common, since it accounts for 1-2% of all cancers, with an incidence of 4 cases per 100,000 population with a higher incidence among women than men (4:1).

There are various types of thyroid cancer: the benign variant is called adenoma and the most common form is follicular, which is capsulated and presents a morphology similar to follicular epithelium. The adenoma differs from carcinoma because it does not invade the capsule or the supplying vessels and generally does not exceed 2 cm in diameter.

There are several types of malignant thyroid tumors: follicular, papillary, medullary and anaplastic. Follicular carcinomas have cytologic features similar to adenomas and are differentiated only by infiltration of the capsule and angioinvasion.

The presence of capsular infiltration may be confused with an irregularity of the capsule which is often present in benign lesions. More conclusive is angioinvasion with the presence of thrombus in the vessel lumen, not to be confused with cellular elements drawn into the lumen during surgical resection.

Pre-surgery diagnosis is based on fine needle aspiration cytology (FNAC), but in 20-30% of cases the cell alteration does not allow a clear identification between follicular adenoma and follicular carcinoma . In these situations, as a precaution, the thyroid is removed and the surgical findings are subjected to histologic examination.

Only 25% of these cases prove to be malignant, while the remaining 75% of patients have undergone an unnecessary thyroidectomy, thus with an associated waste of resources and money, in addition to the fact that the patient will need to take synthetic thyroid hormones for the rest of their life.

For these reasons we have to devise a technique of molecular diagnostics which took into consideration many proteins for use as a tumor marker for thyroid cancer. The most promising of these proved to be galectin-3.

2.5.4 Galectin-3 and Tumors

Galectin-3 belongs to the family of lectins, recognizes and binds various beta-galactosides and is distributed in the membrane, cytosol and nucleus compartments of the cell [107]. It has been shown to play roles in many biological events, such as embryogenesis, adhesion and proliferation of cells, apoptosis, mRNA splicing, bac-

terial colonization and modulation of the immune response [108, 109].

Galectin-3 is expressed in various type of tumor and the intensity of expression depends on tumor progression, invasiveness and metastasis. In most cancers galectin-3 is overexpressed, for example in the cells of pancreatic cancer and in metastases to lymph nodes mRNA levels and protein are much higher than in normal cells [110].

Galectin-3 is not expressed in normal thyroid cells and adenomas (benign lesions), but it is present in malignant thyroid lesions [111, 112].

The fundamental role of galectin-3 in thyroid carcinogenesis has been shown: by inhibiting its expression with oligo antisense into cells of papillary carcinoma there was a marked decrease in malignancy [113], while, normal thyroid cells transfected with galectin-3 cDNA acquired the malignant phenotype [114].

2.5.5 Our Experience

Our goal is the development of a protocol for galectin-3 ESEM immunomarking to use for the differential diagnosis of follicular adenoma/carcinoma of the human thyroid.

To optimize the method and demonstrate the specificity of immunomarking we used a commercial cell line: 3T3 cells (Swiss Albino mouse fibroblasts) in which the expression of galectin-3 was demonstrated [115] and as negative control D16 cells (murine mesoangioblasts).

The method of immunomarking in ESEM was flanked by control techniques: biochemical assay (Western blot) and immunofluorescence confocal laser microscopy.

The two cell lines were subjected to Western blot which demonstrated the presence of galectin-3 in 3T3 and its absence in D16, after which cells were labeled with fluorescent antibodies and analyzed under confocal microscope. The result shows the presence of galectin-3 in both the cytoplasm and cell surface of 3T3 and absence in D16.

The marking protocol was optimized on these two cell lines (antibody concentration, marking time, observation condition, signal level etc.) and it demonstrated the validity of the method [116].

Once a good marking signal with a low background had been obtained, we passed to cell lines closer to the target, taking into account two commercial cell lines of human thyroid: RO-82 (thyrocytes from follicular carcinoma) and FTC-133 (thyrocytes from lymph node metastases). Biochemical assay was also done on these cells, and galectin-3 was identified in these other cell lines. In particular the signal of FTC-133 was more intense than RO-82, thus leading us to assume a role of galectin-3 in the progression of thyroid cancer.

These cell lines were subjected to immunogold labeling using the same protocol as 3T3/D16 (Fig. 2.2a,b). The density of labeling was evaluated by software and an increase in marking in FTC-133 of 25% compared to RO-82 was recorded.

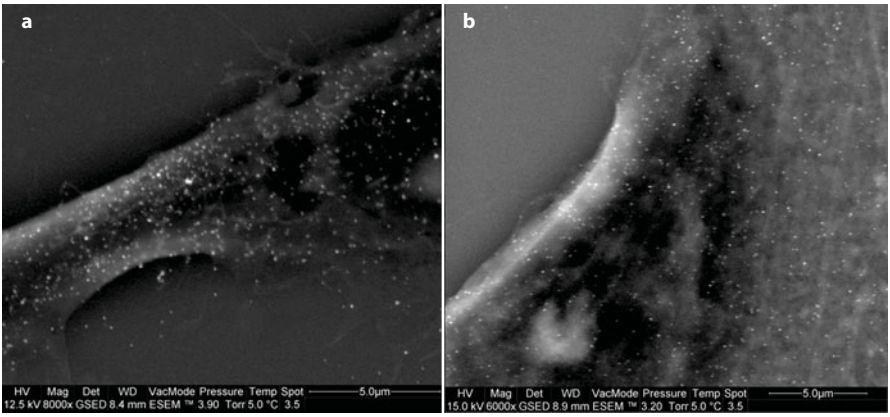

Fig. 2.2 Preliminary experiments to optimize and validate IGL process. **a** FTC-133 cell; **b** RO-82 cell

These data are supported by a field trial, in which we analyzed several FNAC of thyroid lesions processed in ThinPrep.

ThinPrep Processor is an automated slide preparation unit originally used mainly in Pap tests for cervical cancer testing. Now, ThinPrep is also used for non-gynecologic specimens such as the diagnosis of cancers of the lung, bladder, gastrointestinal tract and in the preparation of fine needle aspiration of the thyroid and breast. The ThinPrep Processor utilizes a computerized process and patented membrane technology which controls dispersion, collection, and transfer of diagnostic cells from the sample to the slide [117].

Under the control of the instrument's microprocessor, a gentle dispersion step breaks up blood, mucus, and non-diagnostic debris, and then thoroughly mixes the sample. A series of negative pressure pulses are generated, which draw fluid though a filter to collect a thin, even layer of diagnostic cellular material. The cellular material is transferred to a glass slide and ejected into a cell fixative bath, ready for staining and evaluation.

One recent study defines the ThinPrep technique a valid method for the preoperative cytological diagnosis of thyroid nodules [118].

Were observed 50 samples of thyroid FNA processed with ThinPrep. The cellular material was preserved and displayed well defined morphological features, with no particular damage due to FNA, ThinPrep preparation, methanol fixation and ESEM observation conditions.

The slides showed a great variability in the pattern of cells observed, but most of them (10-15µm in diameter) were represented by typical thyrocytes, the intensity of marking varies from sample to sample and from initial observations seems directly proportional to tumor progression.

Figure 2.3a shows a sample of benign adenoma in which the absence of marking is evident, while Figure 2.3b is a micro-macro follicular adenoma (only *benign* form which is regarded as a potential precursor of cancer) which presents a discrete marking.

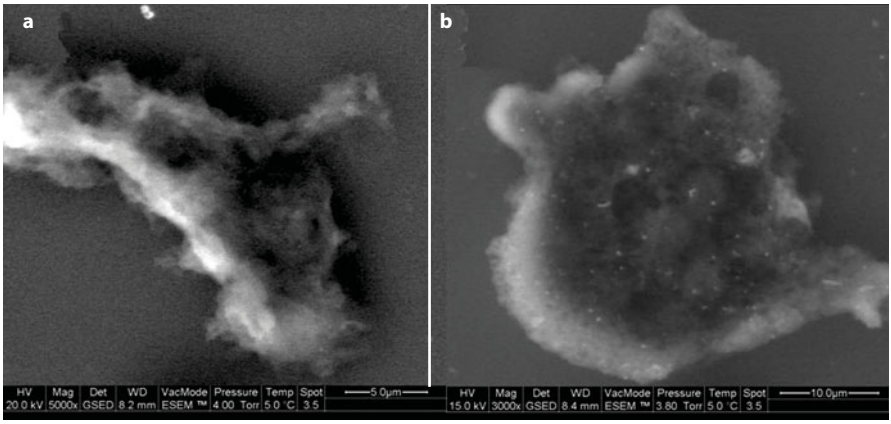

Fig. 2.3 Cell of thyroid FNA ThinPrep processed after IGL. **a** Benign adenoma cell (without marking; **b** micro-macro follicular adenoma cell (discrete marking)

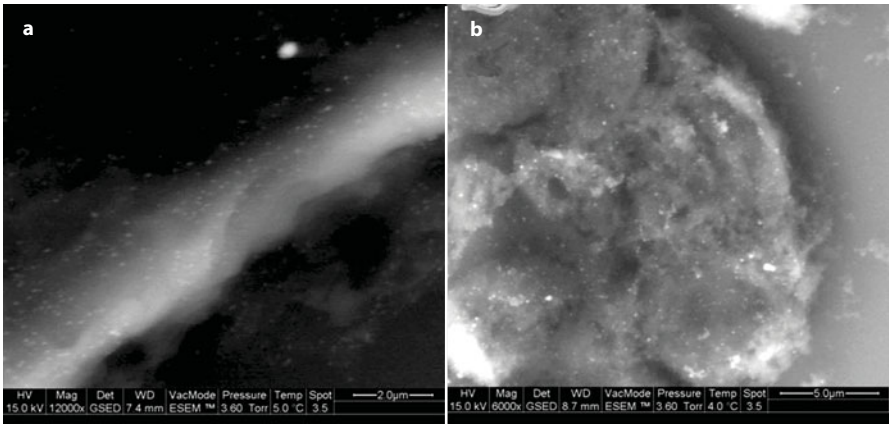

Fig. 2.4 Cell of thyroid FNA ThinPrep processed after IGL. **a**, **b** Follicular carcinoma cell (strong marking)

Figures 2.4a, b are two different samples of follicular carcinoma which show the strong marking of cells, significantly higher than Figure 2.3b.

Clearly, a cytological diagnosis cannot be advanced on the basis of this preliminary result of IGL-ESEM on ThinPrep by FNA. In the future, the analysis of a statistically significant number of FNA-ThinPrep is needed to create a database for pathologists.

The advantage of using the IGL-ESEM compared with classical immunocytochemistry, apart the higher resolution and image definition, is the ability to make a semi-quantitative evaluation of galectin-3 expression on the cell surface and bind to the degree of tumor progression.

Of course this method can be applied to all cancers in which specific surface markers are found.

2.5.6 Conclusions

Current cytological diagnosis of tumors has some inherent limitations related to inadequate sampling and overlapping cytological features between benign and malignant lesions.

This limitation leads, in some cases, to an uncertain diagnosis involving the possibility of an over- or under-treatment in clinical practice.

Our work has demonstrated the possibility of using the IGL in ESEM as an appropriate diagnostic complementary test for discerning uncertain cases in thyroid lesions.

The procedures of ESEM, Thin Prep, the minimum preparation for IGL and the high capacity to collect isolated cells offer a perspective able to supply some help to cytological evaluation. They can be seen as new, original and complementary instruments able to support cytopathologists in their diagnostic work.

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Biomolecular Staging: Reality or Future Perspective?

3

P. Bechi

3.1 Introduction

M. Balzi, P. Faraoni, P. Bechi

The prognosis of patients with colorectal cancer has to date been mainly predicted on the basis of clinical and histopathologic items, with the TNM stage appearing to be the most important.

The TNM staging system and clinicopathological factors have determined clinical decisions in adjuvant therapy for decades and have therefore been improving survival in patients with early-stage tumors. However, the results remain suboptimal. The individual course of the disease within each category of TNM staging is highly variable. Moreover, this system of classification based on topographical extension does not contain information about the biology of neoplasia.

Moreover, the established progression from normal epithelium to dysplastic lesions of increasing morphologic abnormality and finally to locally invasive and metastatic cancer (the *adenoma-carcinoma sequence*) allowed the definition of sequential genetic changes that occur during tumorigenesis. Therefore, there is nowadays the possibility of combining specific molecular and cellular characteristics with tumor stage to obtain a better definition of the disease up to the molecular level.

The definition of tumor biology appears even more important when surgery needs to be associated with chemo- and radiotherapeutic approaches.

In colorectal carcinoma the knowledge of molecular pathogenesis could contribute to defining population groups who are at high risk of recurrence after surgi-

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cal resection with curative intent and to individualize follow-up strategies or adjuvant chemotherapy. Patients with early-stage cancer at low risk of recurrence could be spared the toxicity of systemic therapy, while others at high risk of distant recurrence could obtain maximal benefit if the treatment is chosen on the basis of the biological profile of the tumor.

In other words, due to validated cellular and molecular analysis techniques, the clinical biomarkers could be identified and would cooperate with the TNM staging, so as to improve the therapy [1-4].

The term *prognostic marker* indicates factors related to overall survival or disease free survival. The potential candidates have to respond to two main requisites: the marker must have an independent prognostic meaning and must be evaluated by means of reproducible and standardized methods.

Many of the proposed biomarkers have been analyzed in various studies, but the results are not yet sufficient to demonstrate their true prognostic value with statistical validity and some others have to be investigated with more accuracy and in larger series.

The families of the molecular markers include the genes that influence the apoptosis, the oncogenes, genes correlated to the synthesis and the repair systems of DNA, the inhibitors of cyclin dependent kinases (CDK), and the adhesion and surface molecules.

Other factors that need to be more extensively studied are the DNA content of neoplastic cells, angiogenesis and proliferative activity.

3.1.1 Apoptosis Related Genes

The *p53* tumor suppressor gene is localized on the short arm of chromosome 17 and alterations in the gene locus are the genetic alterations most frequently found in human malignant tumors. p53 protein is involved in proliferation, differentiation, DNA repair/synthesis and programmed cell death. The loss of regulatory activity due to wild type protein provides the proliferative cells with a selective advantage, promotes genetic instability, decreases apoptosis and contributes to deregulated cell growth. p53 status is evaluated with immunohistochemical methods or with the analysis of gene mutations. It has been shown that in colorectal cancer immunopositivity ranges between 30% and 76% of cases, whereas gene mutations are reported in 32% of samples [5]. This discrepancy may be due to mutations that do not produce protein stabilization and consequently may result in a lack of immunohistochemical detectability. Moreover, posttranscriptional stabilization processes can lead to immunohistochemical positivity, without a mutation; hence the determined protein in this case is the wild-type p53. The results of the studies are not uniform especially as far as the prognostic role of p53 alterations is concerned. Many investigations have analyzed the relationship between mutations of p53 gene and unfavorable outcome of colorectal carcinoma, but the conclusions are contradictory. It is worth noting that these studies deal with small numbers of cases, short follow-up

times, absence of multivariate analyses, different methods for determining p53 alterations and different patient populations [6-9].

Bcl-2 belongs to a family of proteins involved in the regulation of cell death and cell survival. Some of these proteins promote apoptosis, whereas others inhibit cell death. Bcl-2 proteins can form heterodimers and the ratio between homo- and heterodimers leads to programmed cell death or to cell survival. The prognostic meaning of the expression of Bcl-2 is controversial: some studies demonstrate that high levels of protein correlate with a good outcome of the disease and that the lack of the expression is associated with metastases and recurrences, but opposite results have also been reported [7]. The assessment of both p53 and Bcl-2 status seems to define a pattern with negative Bcl-2 and positive p53 which seems to be linked to decreased disease free and overall survival [7, 10].

3.1.2 Oncogenes

The family of *ras genes* consists of three homologous members (K-, H- and N-ras) which encode for similar 21-kDa proteins. The membrane-bound ras proteins were thought to play a central role in the transduction of proliferative or mitogenic signals. Ras mutations occur early in the course of the adenoma-carcinoma sequence and activating mutations (in codons 12 and 13) of the K-ras gene are found in 20-43% of colorectal carcinomas. The largest studies (more than 200 patients), dealing with the prognostic value of K-Ras mutations in colorectal cancer, suggest their prognostic role. In the RASCAL multicenter study the effect of mutations in codon 12 was found to be an independent variable predictive of poor outcome [11, 12].

The *DCC* gene (*deleted in colorectal cancer*) is localized on the long arm of chromosome 18 and encodes for a transmembrane cell-surface molecule, showing homology to adhesion molecules. The DCC protein has been suggested to participate in the regulation of cell proliferation and differentiation. In carcinomas, the expression of DCC is frequently decreased. Moreover, chromosomal loss of the DCC gene locus 18q21 (loss of heterozygosity, LOH) has often been reported, thus suggesting a role of DCC gene as a putative tumor suppressor gene.

The studies on colorectal cancer found *18q LOH* in 40-50% of cases and in some clinical research, the allelic loss is reported to be predictive of adverse prognosis, although the results are often controversial [3, 5].

3.1.3 Inhibitors of Cyclin Dependent Kinases (Cdk)

The transition from G1 to S phase of the cell cycle is controlled by complexes constituted by *cyclin D* and *E* and *CdK4* or *CdK6*. The activity of these complexes is inhibited by WAF1/CIP1 (p21, p27, p57) and INK (p15, p16, p18, p19) CDK inhibitors that prevent the progression in the phase of DNA synthesis. The loss of

control of cell-cycle checkpoints is a common event in colorectal tumors and may be an important therapeutic target, although the studies on these important regulatory molecules too frequently examine a single aspect of the phenomenon, thus being inconclusive [13].

3.1.4 Adhesion Molecule Expression

The protein β-*catenin* is involved in the regulation of proliferation and differentiation processes in normal tissues through its role in cell–cell adhesion and gene transcription. In fact, β-catenin, controls cell adhesion at the plasma membrane level and on the other hand is a component of the Wnt signaling pathway and therefore capable of activating the transcription of genes related to proliferation and differentiation. In the adenoma-carcinoma sequence of colorectal carcinogenesis mutations of APC (Adenomatous Polyposis Coli) gene are reported as an early event in up to 85% of sporadic cancers [14]. APC also is a member of the pathway Wnt-β-catenin since the APC protein promotes the degradation of β-catenin in the cytoplasm. Moreover, in approximately 10% of colorectal cancers mutations of the β-catenin gene itself have been reported [15]. Both these alterations can influence the degradation pathway of the protein and promote its stabilization and nuclear accumulation. Nuclear overexpression of β-catenin was reported in advanced colorectal cancers, even if its prognostic significance has not yet been demonstrated [16-18].

3.1.5 Flow Cytometry DNA Content

Flow cytometry analysis allows cell populations containing a normal amount of DNA (diploid) to be distinguished from those containing an anomalous quantity of DNA (not diploid). Different patterns of DNA content are found in tumors: only one clone (diploid or not diploid) or multiple clones with different ploidy. Many studies have analyzed ploidy in colorectal cancer with controversial results regarding its prognostic implications. Not diploid DNA content was found to be associated with poor outcome, but often its contribution to defining prognosis seems not independent from other pathologic factors. Moreover, some studies report no prognostic significance in this biologic marker alone [19]. Flow cytometry determination of DNA content also allows the percentage of cells in the different phases of the cell cycle to be calculated and the S-phase fraction (SPF) of the tumor to then be estimated, which represents a measure of proliferative capability of the neoplastic cells. As a matter of fact, the association of ploidy and proliferation evaluation was able to identify a group of patients with highly proliferating non diploid cancer with a worse prognosis [20].

3.1.6 Conclusions

All the above reported data provide an idea of the complexity of the biological characterization of colorectal cancer, but above all they confirm the absolute necessity to investigate the prognostic implications of the different biomarkers in large series with adequate statistical methods in order to obtain valuable results. This is the first step towards makig the integration of TNM staging and biological aspects a useful tool in cancer treatment.

3.2 Molecular Biology as the Identity Card of Human Tumors

F. Selvaggi, P. Raimondi, P. Innocenti

Genetic and epigenetic alterations in oncogenes, tumor-suppressor genes, cell adhesion molecules and DNA repair genes result in human carcinogenesis. Novel genomic and proteomic technologies have shown promise in providing a molecular taxonomy of tumors. Distinct molecular events have been demonstrated in different human disease such as colorectal, gastric, hepatocellular and pancreatic cancers. The development of colorectal cancer (CRC) from adenoma to carcinoma was first described by Morson et al. [21]. The progression model of CRC follows a sequence of genetic events well characterized by Fearon and Vogelstein, in which the morphological findings correlates with specific and sequential molecular alterations [22]. The earliest genetic change associated with adenomatous polyps is the mutation and/or loss of the tumor suppressor adenomatous polyposis coli (APC) gene. Mutations in the APC gene initiate the adenomatous process, resulting in the clonal growth of a single cell. K-ras mutations have been reported in ~50% of CRC as the genetic event that correlates histologically with adenoma lesions [22]. Additionally, in high-grade dysplastic polyps inactivation of the p53 tumor suppressor gene has been reported and the loss of this gene accelerates the transition from adenoma to carcinoma [23]. CRC results from the cumulative effects of sequential genetic alterations in proto-oncogenes, tumor suppressor genes and DNA repair genes.

Recently, three distinct genetic pathways have been proposed in the development of CRC. First, the mechanism of the adenoma to carcinoma sequence described by Vogelstein which considers the chromosomal instability (CIN) or loss of heterozygosity (LOH) pathway [22]. This pathway accounts for roughly 80% of all sporadic CRC and involves oncogenes such as K-ras, C-erb2, C-myc or tumor suppressor genes such as APC, delete in colon cancer (DDC), and p53. The second distinct genetic pathway is termed microsatellite instability (MSI) and accounts for about 20% of all sporadic CRC [24]. Mutations in a mismatch repair gene result in the failure to correct replication errors that occur during DNA synthesis. The predisposition to Lynch syndrome, a hereditary nonpolyposis CRC, is caused by a mutation in one of several DNA mismatch repair genes, most commonly in MLH1, MSH2 or

MSH6 [25]. The CpG island methylation phenotype (CIMP) is the third and most recently identified pathway involved in CRC. It involves hypermethylation of gene promoter regions, which results in silencing of these genes. CRC are characteristically sporadic (non familial) and have a distinct clinical profile that includes proximal tumor location, female sex, older age, high tumor grade, wild-type TP53, higher BRAF and K-ras mutations, and frequent MSI [26].

Currently, 5-fluorouracil (5-FU) is the chemotherapeutic agent of first choice for treatment of CRC in combination with leucovorin and with irinotecan or oxaliplatin for metastatic disease. The active metabolite of 5-FU, inhibits thymidylate synthase (TS), the enzyme essential for the DNA synthesis pathway. TS expression is a predictive marker of advanced CRC in patients receiving fluoropyrimidine-based chemotherapy [27]. In 2008, Jover et al. evaluated the response of mismatch repair MMR defective colorectal cancer to adjuvant 5-FU chemotherapy in patients prospectively followed during 5 years. They demonstrated that CRC patients in stage II or III exhibiting competent mismatch repair tumors obtained an important benefit from 5-FU based chemotherapy, improving the overall survival and disease-free survival to almost 20%. In contrast, patients affected by CRC with a deficient MMR status showed poor prognosis. The clinical relevance was that adjuvant 5-FU based chemotherapy may not be useful in patients with MMR-defective colorectal cancer with stage II or III disease [28]. K-ras mutations have emerged as a major predictor of resistance in patients with metastatic colorectal cancer treated with cetuximab or panitumumab [29]. The addition of cetuximab to first-line FOLFOX (folinic acid, fluorouracil, and oxaliplatin) or FOLFIRI (folinic acid, fluorouracil, and irinotecan) chemotherapy did not ensure benefits in patients with tumors carrying K-ras mutations, although those patients can clearly benefit from chemotherapy alone [30, 31]. Starting from these observations based on a complex molecular machinery, additional studies are advocated to better define the molecular alterations and design well-defined therapeutic strategies.

Specific genetic and epigenetic alterations have been studied in gastric cancer (GC) tumorigenesis. Inactivation of various genes including p16, MLH1, cadherin1 (CDH1), RARβ2, pS2 and RUNX3 by DNA methylation is involved in two distinct major molecular pathways [32]. Hypermethylation of the p16 and MLH1 promoters is associated with the intestinal type, whereas hypermethylation of the CDH1 and RARβ2 promoters predominantly occurs in diffuse type gastric carcinoma. An adenoma/carcinoma sequence is found in about 20% of gastric cancers with APC mutations. Several proto-oncogenes, including C-met, K-sam and C-erb2 are activated in GC. Over-expression of C-erb2 confers a poor prognosis and liver metastasis. Mutations in E-cadherin gene, a cell adhesion molecule, occur in 50% of diffuse type gastric carcinomas and are involved in the development of diffuse and scirrhous-type gastric cancer. More importantly, reduction of p27 expression in frequently observed in advanced GC while it is well preserved in 90% of gastric adenomas and 85% of early cancers. Loss of p27 expression and gain of cyclin E promotes progression and metastasis of GC [32].

The most important factor implicated in gastric carcinogenesis is genetic instability including microsatellite instability (MSI) and chromosomal instability. GC

express a spectrum of growth factors, cytokines including TGF-α, TGF-β, EGF, PDGF, IGF, IL-1α, IL-6, IL-8. These growth factors and cytokines organize the complex interaction between cancer cells and stromal cells and play a key role in morphogenesis, invasion, neovascularization and metastasis [32]. Diffuse GC syndrome is an autosomal dominantly inherited GC disease caused by germline mutations in CDH1, the gene encoding E-cadherin. This syndrome can be both accurately predicted and successfully prevented by testing CDH1 gene. This germline mutation has been identified in approximately 40% to 50% of well defined hereditary diffuse gastric cancer (HDGC) families.

In hepatocarcinogenesis, accumulation of genetic and epigenetic changes occur during initiation, promotion, and progression of this disease. Hepatocellular carcinoma (HCC) displays different genomic alterations, including chromosomal instability, CpG methylation, DNA rearrangements associated with HBV integration, DNA hypomethylation, and microsatellite instability [33]. Variability in the number and types of genetic changes has also been observed geographically, and may be dependent upon the etiology of the tumor (viral, chemical or both).

Several pathways are being studied extensively in HCC to identify potential biomarkers and molecular targets. The Wnt signaling pathway is involved in HCC arising from HBV/HCV infections and alcoholic liver cirrhosis [34]. Studies have reported that p53 mutations and inactivation play a critical role in HCC and detection of mutant p53 in plasma samples serves as a potential biomarker for aflatoxin exposure and presence of liver cancer [34]. There is evidence that mutation R249S gene occurs as the result of mutagenesis by aflatoxin in a context of HBV chronic infection [35]. Disruption in the Rb (retinoblastoma) pathway in HCC is similar to that observed in various cancers. Also intracellular mitogen-activated protein kinase (MAPK) family has been demonstrated to play a central role in hepatic carcinogenesis. HBV, HCV, and hepatitis E virus modulate MAPK signaling by targeting multiple steps along the signaling pathway [34]. In human HCC, the expression levels of Spred protein (Sprouty-related protein with Ena/vasodilator-stimulated phosphoprotein homology-1 domain), an inhibitor of the ras/raf-1/ERK pathway, are deregulated [33]. This finding shows a direct correlation of MAPK-ERK pathway activation and HCC progression. H-ras, N-ras, K-ras4A, and K-ras4B are small GTP-binding proteins that function as molecular switches to influence cell growth, differentiation and apoptosis [36]. Single point mutations in codon 13 of H-ras, codon 12 of N-ras, and codon 61 of K-ras were originally observed in HCC caused by various chemicals such as N-nitrosomorpholine, bleomycin, 1-nitropyrene, and methyl (acetoxymethyl) nitrosamine [34]. It has been suggested that the ras pathway is important in HCC of rodents but not human HCC based on the low mutation rate of ras in humans. However, it was reported that RASSF1A and NORE1A, members of the RASSF family of ras inhibitors, are inactivated in human HCC, demonstrating the role for the ras pathway in liver cancer [36]. The molecular dynamics of HCC can also be influenced by proteins and cellular factors of other signaling pathways. For example, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) play important roles in HCC development [33]. Recent data indicated that sorafenib, a multikinase inhibitor with anti-angiogenic, pro-apoptotic and raf kinase

inhibitory activity, is the single effective therapy in prolonging survival of patients with advanced HCC [36]. Median survival and the time of radiologic progression were nearly 3 months longer for patients treated with Sorafenib than for those who received placebo [37].

Inflammation is inherently associated with cancer and a number of cytokines are involved in promoting HCC development and progression, especially during infection with hepatitis viruses [33]. Therefore, modulating the expression of cytokines with the administration of cytokine inhibitors might be extremely useful in controlling HCC progression. The use of inhibitors of EGF receptor and TGF blocks the development of HCC in rat liver [36]. Specific areas of the genome appear unstable in HCC: gains of 1q21-23 and 8q22-24 were identified as genomic events associated with the early development of HCC in a genome-wide study of chromosomal aberrations of 158 HBV-associated HCC [34]. Frequent promoter hypermethylation and subsequent loss of protein expression has also been demonstrated in HCC at tumor suppressor gene, p16, p14, p15, SOCS1, RIZ1, and E-cadherin.

Several tumor biological factors such as DNA aneuploidy, high tumor cell proliferation index, high telomerase activity, and mutation of the p53 gene have been associated with increased risk of postoperative tumor recurrence in HCC [36]. Molecular analysis of 63 selected genes can predict the response of HCC patients with major portal vein tumor thrombi to combination chemotherapy with 5-FU and interferon-alpha, suggesting that the gene expression profile predicts outcome not only in surgical patients but also in patients with advanced HCC who received medical care [34].

Angiogenesis in HCC has been found to correlate with the presence of metastasis. VEGF promotes the growth, migration, and morphogenesis of vascular endothelial cells and increases vascular permeability [38]. The elucidation of the mechanisms of angiogenesis is of importance because anti-angiogenic agents are now available and may be of potential benefit in patients with HCC [36]. Use of bevacizumab, an anti-vascular endothelial growth factor monoclonal antibody, in unresectable HCC has showed overall survival rate of 53% at 1 year, 28% at 2 years, and 23% at 3 years [38].

Molecular genetic analysis has provided evidence that pancreatic duct lesions are the precursors of the infiltrating adenocarcinomas of the pancreas. The genetic progression model of pancreatic ductal adenocarcinoma (PDAC) provides strong support for a ductal origin of this malignancy. Moreover, observations from animal models indicate that putative pancreatic stem cells and transdifferentiated endocrine or exocrine cells might be involved. Although it seems that the ducts are a main source of pancreatic neogenesis, each pancreatic compartment shows the capacity for assuming a dedifferentiated duct-like phenotype with multipotent differentiative capacity [39].

In 1994 Klimstra and Longnecker proposed a classification system for hyperplastic non-invasive lesions named pancreatic intraepithelial neoplasia (PanIN). This system, based on morphological features, defined a progression model for pancreatic neoplasia [40]. The earliest precursor lesions, PanIN-1A and -1B, characterized by elongation of ductal cells with mucin production and, in the case of PanIN-

1B, with papillary instead of flat architecture, are found in up to 40% of non malignant pancreata. These lesions with minimal cytological and architectural atypia have been shown to harbor activating point mutations in the K-ras oncogene and to overexpress the HER-2/neu gene product [39, 40]. These alterations in K-ras and HER-2/neu are believed to be early genetic events in the development of pancreatic neoplasia. Inactivation of p16 tumor suppressor gene appears to occur later [39, 40]. As PanIN lesions progress, they acquire moderate (PanIN-2) and severe nuclear abnormalities with abnormal mitoses and budding of cells into the lumen (PanIN-3, formerly known as carcinoma in situ). PanIN-3 lesions are present in 30%-50% of PDAC. Loss of p53, DPC4 and BRCA2 tumor suppressor genes appears to occur late in the development of pancreatic neoplasia [40]. However, in contrast to K-ras, HER-2/neu and p16, these gene abnormalities are associated with significant duct cytologic and architectural atypia. Mutations of K-Ras, CDKN2A, TP53, BRCA2 and SMAD4/DPC4 contribute to the biological characteristics and evolution of the disease. Among the genetic lesions that are linked to familial PDAC are germline mutations in CDKNN2A, BRCA2, LB1 and MLH1 [39]. Loss of CDKN2A function occurs in 80-95% of sporadic PDAC. CDKN2A loss is generally seen in moderately advanced lesions that show features of dysplasia. The TP53 tumor-suppressor gene is mutated in more that 50% of cases. TP53 mutations arise in later-stage PanINs that have acquired significant features of dysplasia. SMAD4 seems to be a progression allele for PDAC, as its loss occurs only in later-stage PanINs and represents a predictor of decreased survival. PDAC overexpresses EGF-family ligands, such as transforming growth factor-α (TGF-α) and EGF and receptors (EGFR, ERBB2). The EGFR and ERBB2 induction occurs in low-grade PanINs [39]. These molecular observations form the foundation for a progression model for pancreatic cancer [39, 40]. This does not imply that all PanINs progress to infiltrating carcinomas. Additional studies are needed to examine such possibilities.

Molecular studies have demonstrated the involvement of multiple signal transduction pathways, such as the RAS-BRAF-MEK ERK signaling cascade (the so-called MAP kinase pathway) in thyroid cancer. BRAF mutation is associated with a more aggressive phenotype and radioiodine-refractory behavior. In addition, gene rearrangement involving RET or the activating of RAS family protooncogene are essential for papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). Additionally, the over-expression and/or hyperfunctioning of vascular endothelial growth factor (VEGF) and its receptors, hepatocyte growth factor (HGF) and its receptor (MET), platelet derived growth factor (PDGF) and its corresponding receptors have been found in these tumors. Kinase inhibitor molecules represent the first-line candidates for the therapy of refractory tumors. The histone deacetylase (HDAC) inhibitors and retinoid derivates are encouraging tools in pharmacologic strategy [41]. Adenomas can be differentiated from follicular carcinomas by genes such as PEG10, PLCB4, FNB1, ID4, and DUSP1 [42]. Some studies have found that the phosphatidylinositol 3-kinase (PI3K)/AKT pathway plays an important role in thyroid tumorigenesis, particularly in the progression from low to high grade tumor [43]. The molecular pathogenesis of anaplastic thyroid cancer (ATC) includes mutations in BRAF, RAS, PIK3CA, TP53, PTEN, and APC genes. The

expression of EGFR in ATC makes it an attractive target for drug development, using monoclonal antibodies such as cetuximab and kinase inhibitors such as gefinitib and imatinib [44].

Molecular profiling studies have designed some aspects in the biological behavior of breast cancers. Five major subtypes of breast cancers have been defined: basal like, luminal A, luminal B, HER2+/ER-, and normal breast like [45, 46]. Luminal tumors are more differentiated, hormone receptor-positive, showing a better outcome. HER2+ tumors have amplification of the ERBB2 oncogene and respond to therapy targeting this receptor kinase. Basal-like tumors are poorly differentiated, lack hormone receptors and HER2, with a worse clinical outcome. The current accepted view of breast tumor progression assumes the hypothesis of a gradual step-by-step transition of ductal hyperproliferation to in situ then invasive and finally metastatic carcinomas. In this model, ductal carcinoma in situ (DCIS) is considered the obligate precursor of invasive ductal carcinomas [46]. Well differentiated breast tumors were more frequently hormone receptor-positive and negative for HER2, p53, and Ki67 compared with poorly differentiated tumors. Current models explaining inter- and intratumoral diversity are the cancer stem cells and the clonal evolution hypothesis [46]. The clonal evolution model supports the role of genomic instability, resulting in the continuous acquisition of new somatic changes. The number of genes mutated in breast cancer is high and concerns the PI3KCA/AKT/PTEN, TP53 and NF-kB pathways. The PI3K pathway is, therefore, an attractive target for breast cancer therapy [47]. Unfortunately, the transition mechanisms of DCIS to invasive carcinoma is still not well understood, and crucial genetic events have been identified only in part in breast cancer patients [46].

Thanks to the tools of molecular research, our understanding of cancer progression has significantly increased during the last decades but the translating these findings into clinical practice still remains a challenge.

3.3 Interventional Timing According to New Insights in Basic Research

P. Innocenti, F. Selvaggi, D. Risio

The surgical approach and its interventional timing have been strongly influenced by current scientific knowledge and modern multidisciplinary therapeutic options have been defined according to new biomolecular insights. Nowadays, general consensus has been expressed about the role of prophylactic surgery, particularly in hereditary cancer syndromes and in tissue or organ regeneration. One of the most important consideration is the timing of thyroidectomy in children with mutated RET allele [48]. Before 1994 there was no genetic testing available for screening RET mutations and affected family members underwent thyroidectomy after the identification of pathologic increased levels of serum calcitonin. Today it has been generally accepted to perform prophylactic surgery in patients identified by genetic analysis [48]. Most surgeons advocate that the ideal age for prophylactic surgery for

patients carrying a mutation in exon 11 codon 634 is as early as age 5 [49]. Regarding the need of lymphadenectomy there is no consensus. There are also surgeons who routinely do bilateral cervicocentral lymphadenectomy in their prophylactic operations [49]. DNA analysis used to identify risk for MEN 2B have shown germline mutation in codon 918 of the RET protooncogene. In these patients MTC tends to present at a very early age and appears multifocal, bilateral and more virulent [48]. In addition, RET mutations were found at codons 609, 611, 618, 620, 634, but no statistical evidence that the presence of persistent or recurrent medullary thyroid carcinoma was dependent on the specific genetic alterations [50].

If we consider hereditary breast cancer development, current data coming from basic research confirm that the genetic predisposition is 5-10% [48]. Women with BRCA1 or BRCA2 mutation have a cumulative risk of invasive cancer ranging from 55 to 85% and of invasive epithelial ovarian cancer ranging from 15 to 65%. The risk of developing breast cancer increases near the age of 25 years. The identification of breast cancer susceptibility genes BRCA1 or BRCA2 was performed in 1994 and 1995 respectively according to the evidence of premature truncation of the BRCA1 or BRCA2 protein. Modern prophylactic surgery for women at high risk of breast cancer, according to the molecular tests, include total bilateral mastectomy (TBM) without axillary lymph node dissection, skin-sparing total mastectomy, and subcutaneous nipple-sparing mastectomy, reconstruction with artificial breast implants or tissue reconstruction options using transverse rectus abdominis flap and the latissimus dorsi flap. A final procedure being discussed is areolar-sparing mastectomy [48, 51]. The efficacy of prophylactic TBM in reducing the incidence of breast cancer at three years of follow-up has been demonstrated [52]. Prophylactic TBM reduced the risk of breast cancer by 95% in women who also had a risk-reducing salpingo-oophorectomy and by 90% in women that had intact ovaries [48]. Kurian et al. recently demonstrated that prophylactic TBM at the age of 25 instead of 40 offers minimal incremental benefit (1% to 2%). Prophylactic surgery is a highly personal decision and the protective effects of surgery must be weighed according to possible complications and psychological problems.

Advances in molecular diagnostic techniques have changed the landscape of Lynch syndrome, the hereditary syndrome that predisposes to colorectal cancer. The common mutations concern the mismatch repair (MMR) genes, in particular *MLH1*, *MSH2*, *MSH6* and *PMS2*. The benefits of prophylactic hysterectomy and bilateral salpingo-oophorectomy in preventing gynecologic cancers in women with Lynch syndrome after the age of 35 have been demonstrated [53]. Colorectal cancer occurs in 78% to 80% of MMR mutation-positive patients at a mean age of 46 years. Endometrial cancer occurs in 43%, gastric cancer in 19%, urinary tract cancer in 18% and ovarian cancer in 9% of Lynch syndrome affected individuals [51]. Molecular analysis indicates that each mutated MMR gene has a distinguishable cancer risk profile with different clinical management. Female MSH6 mutation carriers have a lower colorectal cancer risk and higher risk for developing endometrial carcinoma reported to be around 71%. For these patients biennial colonscopic surveillance at an age of 30 years has been advocated and prophylactic surgery for gynecologic hereditary cancer may be considered from an age of 45 years or once

childbearing has been completed [54]. Inherited colorectal cancer comprises familial adenomatous polyposis (FAP) caused by mutations in the tumor suppressor adenomatous polyposis coli (APC) gene. Three surgical options are currently proposed for FAP patients: total proctolectomy with permanent ileostomy, total colectomy with ileorectal anastomosis, and proctolectomy with ileal pouch anal anastomosis. The risk of incomplete excision due to uncontrolled rectal polyposis may be estimated by the specific location that causes APC mutation [51]. Mutations located at codons 1250 and 1464 had a 6.2-fold increased risk of rectal cancer compared with those with mutation before codon 1250 or after codon 1464. The choice of the optimal surgical treatment depends on several factors. Its interventional timing is still discussed and no general consensus has been expressed. Differences in phenotype expression of cancer mandate different surgical approaches in FAP or Lynch syndrome, including the type and timing of colorectal surgery. Although the importance of genotype-phenotype relations, the considerations in choosing surgical therapies have to underline primarily the clinical outcome and associated quality of life [51]. Prospective trials to determine the efficacy of surveillance and surgical therapies in FAP or Lynch syndrome families are needed.

Patients with evidence of E-cadherin (CDH1) germline mutation in the setting of a family history of hereditary diffuse gastric cancer (HDGC) must be considered as candidates for prophylactic total gastrectomy (PTG) [55]. In a retrospective study, Hebbard et al. demonstrated that patients who underwent PTG had a truncating mutation of the CDH1 gene with mutation 2398delC of exon 15 and 1189A>T in exon 9 of CDH1. Interestingly, preoperative mucosal biopsies revealed the disease only in 9% of cases while evaluation of surgical specimens showed evidence of carcinoma micro-foci in 96% of cases [56]. If the CDH1 test is positive, PTG should be considered. However, the published data supporting this evidence are still limited.

Findings in molecular research have provided new impetus for the use of stem cells in the treatment of gastrointestinal and liver diseases. Interestingly, administration of autologous CD133+ bone marrow stem cell to the liver significantly increases hepatic regeneration [57]. Portal vein embolization may be easily associated with CD 133+ bone marrow stem cell administration to augment hepatic regeneration in cases of large hepatic malignancies. Patients who received bone marrow stem cell portal application and portal vein embolization had mean daily hepatic growth rates 2.5 fold higher compared with subjects that received portal vein embolization alone [57]. The intravenous administration of stem cells induces proliferation of hepatocytes in time for safe oncologic surgical resection. The implication of rapid liver growth suggests the possibility of enrolling patients with an insufficient remnant liver volume to curative surgical resection after the selective exclusion of the tumor mass for example by using portal vein embolization procedures or other interventional strategies and to reduce the time to surgery by an average of 18 days [58]. Preliminary data coming from molecular biology are encouraging but liver regeneration is a complex process and much additional work is needed to define factors required in liver cellular differentiation as well the safety, efficacy and tolerability of stem cells.

In pancreatic ductal adenocarcinoma (PDAC) molecular analysis has shown that

around 10% of pancreatic cancer is hereditary in origin. Some studies have evaluated matrix metalloproteinase-7 (MMP-7) as a biomarker, measuring its level in plasma and pancreatic juice, with the observation that combined MMP-7 and CA19-9 assessment gave a positive predictive value of 100% [59]. Combination molecular analysis able to detect DNA abnormalities characteristic of PDAC (K-*ras*, *p*16 and *p*53) in pancreatic juice increase the discrimination between patients with malignant and benign disease [60]. Prophylactic pancreatectomy based on today's molecular knowledge is not indicated and the screening and surveillance of high risk members of FPC using ultrasound and endoscopic retrograde cholangiopancreatography is currently an effective method. Patients who have evidence of PanIN III lesions (carcinoma in situ) might be indicated the option of pancreatectomy. There is an urgent need to discover biomarkers and to improve early diagnosis in high-risk PDAC patients.

Tissue engineering is the new frontier of plastic reconstructive surgery [61]. Recently attention has been focused on adipose-derived stem cells (ASCs) [62]. Breast augmentation is one of the most frequently performed cosmetic surgeries. Nowadays most surgeons use artificial implants or augmentation with lipoinjection, with various complications such as capsular contracture or postoperative atrophy. To overcome these problems a novel strategy known as cell-assisted lipotransfer (CAL) which uses autologous ASCs and lipoinjection techniques, was developed. Recent results demonstrated that breast circumference increased by 4 to 8 cm at 6 months in the observed cases [63]. Compared with breast augmentation using implants of the same size, augmentation with CAL showed a lower height but a more natural contour of the breast. Further improvements in stem cell biotechnologies may lead these experimental procedures as future gold standard treatments in plastic reconstructive surgery.

Molecular biology is modifying surgical approaches and the interventional timing, but further improvements in translational research have to be reached to define individualized treatment especially in sporadic malignancies.

3.4 Cellular Biology: A Way of Predicting Cancerogenic Progression, Prognosis and Response to Adjuvant Treatment

M. Balzi, P. Faraoni, A. Taddei

In adults, the epithelia are steady state systems with a continuous production of cells that substitute the differentiated cells when they become senescent and die. Proliferation is a strictly controlled process and in the course of the cell cycle, serious DNA alterations are recognized and driven to apoptosis at different checkpoints. This mechanism preserves the integrity of the cell population.

Many physical, chemical and biological environmental factors induce cell alterations that can modify the percentage of proliferating cells. Proliferating cells are very sensitive to DNA damage because during DNA synthesis the molecule is no

longer wrapped around histones and it is exposed to injury with a greater frequency than differentiated cells.

In the 1990s the hypothesis *mitogenesis stimulates mutagenesis* was formulated and this led to verifying whether different agents able to influence proliferation are involved in the steps of carcinogenesis.

Our research group has been studying human gastric epithelium in patients with increased risk of gastric cancer. We analyzed two models: (1) subjects affected with functional dyspepsia tested for the presence/absence of *Helicobacter pylori* (Hp); (2) partial gastrectomized patients with different surgical reconstruction. In both groups proliferative activity was determined on multiple biopsies collected from different sites of the stomach.

Biopsies were placed immediately on a rigid support to maintain orientation of the foveolae and incubated in a culture medium containing ^{3}H-thymidine. After one hour the samples were washed several times in cold saline, fixed in Carnoy's solution and embedded in polystyrene. Sections 3 μm thick were cut oriented according to the longitudinal axis of the pits and were submitted to autoradiography. The cells constituting the epithelial row were counted from the neck to the mucosal surface. Moreover, both the number and the position of labeled cells were determined for each left side of the well aligned pits. Mean value of the total cell number, labeling index, mitotic index and the localization of labeled cells were calculated in normalized pits obtained after counting 50 foveolae of each patient. In Hp negative subjects the results were different in the corpus respect to the antrum, where the number of epithelial cells was more than double. The percentage of labeled cells in the pits was higher in the central part and lower in the upper part near the gastric lumen. Hp positive subjects showed a statistically significant increase in the total cell number both in the antrum and in the corpus and a significant increase of thymidine labeling index (TLI) only in the corpus when compared to Hp negative patients. The distribution of labeled cells along the pits in the antral mucosa appeared slightly different in the Hp positive and Hp negative subjects; more specifically, Hp positive patients showed an upward shift of the maximal frequency of labeled cells in the pits when compared with Hp negative subjects. A shift of the proliferative compartment towards the luminal surface was observed in Hp positive patients when severe gastritis was present. These results show that the Hp infection is associated with modifications of the proliferative compartment of the gastric mucosa and this is especially evident in patients with gastritis. The increase and shift towards the surface of proliferating cells expose cycling cells to mutagenic and carcinogenic molecules present in the gastric lumen. The action of these factors and the chronic inflammatory condition could explain the increased risk of cancer in patients affected with Hp infection [64].

The second model to explain the increase of carcinogenesis in the stomach is represented by subjects submitted to partial gastrectomy for benign ulcer disease at least 12 years earlier. In three homogeneous groups of patients, with Billroth II (BII), Billroth I (BI) or total biliary diversion reconstruction (TBD), the relationships between bile acid reflux, gastric histology and cell proliferation were studied. The concentration of bile acids in the stomach remnant was higher in BII patients

than in BI and bile reflux was absent or very low after TBD. Experimental studies have previously demonstrated that bile acids are able to stimulate cell proliferation in the ileal and colonic mucosa and for this reason they are thought to play a role in the initiation and promotion steps of intestinal carcinogenesis. For each patient multiple biopsies were collected in three different areas of the gastric stump: immediately perianastomotic, 2 cm from the anastomosis and at the level of the proximal body. As described above, TLI and the distribution of labeled cells were determined for each biopsy. Moreover, the concentration of bile acids in the gastric aspirates was assayed. The highest degree of foveolar hyperplasia was observed in perianastomotic mucosa of BII patients with a progressive decrease with increasing distance from the anastomosis. Hyperplastic changes were less evident in BI and virtually absent in TBD patients. Total pit cell number and TLI were significantly higher in perianastomotic biopsies of BII subjects. These results showed that patients with the highest concentration of bile salts in the stomach had a more severe degree of foveolar hyperplasia and the highest values of proliferation parameters, whereas the lowest levels of all the parameters were found in TBD patients. An evident shift towards the luminal surface of the proliferative compartment was observed in BII perianastomotic biopsies in which a significant increase of labeled cell frequency was found along the whole length of the pit and particularly in its upper half. These findings demonstrate a close relationship between postgastrectomy bile reflux, foveolar hyperplasia and cell kinetic parameters. Histologic and cell kinetic findings are probably the expression of gastric adaptation to chronic bile reflux exposure. The type and entity of cell kinetic modifications and its relationships to foveolar hyperplasia and bile salt reflux may be an important biological tool in postgastrectomy follow up in order to identify high risk patients. Moreover, they could help to clarify the pattern of gastric carcinogenesis [65].

Our other study which we started a few years ago aims to assess the possible prognostic role of some biological/molecular markers in patients with colorectal cancer. The cases were recruited on the basis of strictly controlled criteria (only primary carcinomas, no systemic or local treatment before surgery and no distant metastases at surgery).

Fragments of tumors and normal mucosa were used for the evaluation of the immunohistochemical expression of proteins influencing apoptosis, such as p53 and Bcl-2, adhesion molecules like β-catenin and Ki-67 antigen as proliferation index. Moreover, for each case the flow cytometry DNA content was assayed and ploidy and cell cycle distribution of the neoplastic cells were determined. Some preliminary study conclusions concerning the biological characteristics of 400 colorectal adenocarcinomas can preview the proportion of non diploid tumors is 60% and the frequency of non diploid tumors is higher in the distal colon and lower in the proximal colon. There is a progressive increase of S-phase fraction (SPF) levels comparing diploid and the different classes of non diploid tumors. P53-positive cases are 82.5% and Bcl-2 positive tumors are 49.6%. The expression of the antiapoptotic protein is more frequent $p<0.05$) in stage I than in stage II and stage III tumors. As regards β-catenin immunoreactions, the three cell compartments where the protein can be found were separately evaluated: location in the membrane was registered in

89.7% of the cases, in the cytosol in 80.6% and the nucleus in 54.0% of carcinomas. One interesting finding is the co-expression of β-catenin in the nucleus and in the cytoplasm and the corresponding lack of expression at the membrane level. The presence of the protein in the nucleus of neoplastic cells is more prevalent in the distal colon when compared with the proximal colon.

The aims of the study are to verify the prognostic independent predictive value of biological markers and to explore the possibility of a biologic staging system which could be complementary to conventional staging so as to better define the severity of the disease. These studies seem to be at a more advanced stage for other cancers [66, 67].

At present we have assayed the ability to predict prognosis for single biological parameters in univariate analysis dividing the cases on the basis of TNM stage. In particular, the group of patients with stage II carcinoma was chosen and the overall survival (OS) probability was analyzed for the different expression of biological markers. As an example, the pattern of OS in the groups with and without nuclear β-catenin expression is given in Figure 3.1. Patients with nuclear β-catenin positive tumors have lower levels of OS (73.2%) when compared with the group in which nuclear β-catenin is not expressed (87.1%). This datum seems perfectly understandable if we consider that the translocation of β-catenin into the nucleus corresponds to a change in its function, from adhesion molecule to transcriptional activator, therefore the nuclear expression could be seen as a sign of aggressiveness of the neoplastic cell population.

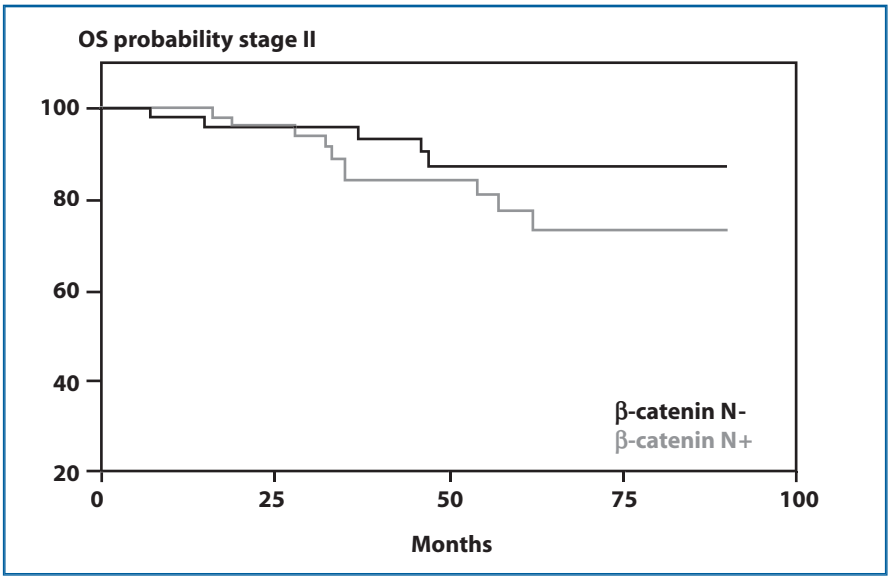

Fig. 3.1 Overall survival probability in stage II patients with nuclear β-catenin negative and positive colorectal tumor

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Technology for Biotechnology

4

F. Rosso, M. Barbarisi, A. Barbarisi

4.1 Nanotechnology and Nanofabrication

Nanotechnology is an emerging science that studies how to control matter on an atomic and molecular scale. Generally nanotechnology deals with structures of the size of 100 nanometers or smaller in at least one dimension, and involves developing materials or devices within that size. Nanotechnology ranges from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale to investigating whether we can directly control matter on the atomic scale.

Nanotechnology has the potential to create many new materials and devices with a wide range of applications in fields such as in medicine, electronics and energy production. However, nanotechnology raises many of the same issues as with any introduction of new technology, including concerns about the toxicity and the environmental impact of nanomaterials.

Nanofabrication is a set of techniques which allow the construction of objects, devices and *small* materials with nanometer dimensions, where *small* means a better outcome, more economic, faster operations and higher performances. In a broader meaning, nanofabrication is one of the different disciplines of precision engineering which takes advantage of such techniques for the realization of small 3D structures which can find applications in different sectors of the electronics industry, and now in the biomedical field too. The classical technique used to produce geometrically patterned devices with nanometric dimensions is lithography which is based on the transfer of a determined pattern copied onto solid materials.

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Over the years, lithography has developed into photolithography, X-ray lithography, and ionic or electronic beam lithography [1].

Usually these expensive techniques are used for the fabrication of electronic microcircuits or in semiconductor technology, although on rigid materials such as metals, quartz and glass. Recently, alternative not-photolithographic techniques have been developed, such as microcontact printing, replica molding etc., which are called soft lithography [2]. The characteristic of soft lithography is to replicate, rather than fabricate, the pattern. These methods are cheaper than the photolithographic techniques, the procedure is quite simple and it requires no special equipment. The main advantage is the application of such fabrication techniques to different kinds of materials which may be less rigid, such as polymer materials [3].

The possibility of adapting nanofabrication techniques to different kinds of materials, including the polymers which form the basis of many biomaterials, has made them useful for the realization of nanostructured devices for the biomedical field. This means the fabrication of medical devices which may show better biocompatibility, biointegration and functional properties [4]. Many tolerance problems have to be faced when a prosthesis is implanted in any part of the body: these arise from the interaction between the prosthesis surface and the physiological environment with which it comes into contact.

The realization of nanostructured surfaces means building intelligent complex biomaterials able to guide, accelerate or condition determined biological processes. It is well known that the surface control of cell behavior such as adhesion, proliferation, movement and differentiation plays an important role in tissue formation and repair [4]. The migration ability (the cell's ability to move) more than any other factor plays an important role in many biological processes such as tissue repair, artificial prosthesis epithelialization and vessel endothelialization. The control of cell morphogenesis is a fundamental requirement for obtaining advanced tissue devices, that is to say artificial devices on which cells can spontaneously and precisely organize and mimic natural tissue morphology and functionality. The presence of microdomains and nanodomains on a surface may allow the control and manipulation of two external signals – the cell-substrate and the cell-cell interactions – to produce a unit of highly oriented and differentiated cells able to organize themselves into a tissue.

The realization of devices having on their surface topographic and/or chemical nanometric domains means being able to comprehend the interaction occurring between the prosthesis surface and the first factors with which it comes into contact, such as physiologic liquid proteins, blood cells and tissue cells.

The *cell guide* depends on the characteristics of the structured surfaces in terms of dimensions of the domains with different topography and/or chemical composition. The domain dimensions can be as big as those of the biological entity with which they interact. In fact, we have to be aware of the average dimensions of a cell, which vary within a range of 10-20 micron, but the cell surface also has functional domains which can be 50-100 nm [5].

Most studies aim at the realization of surfaces with only topographic domains with different kinds of geometric configurations [6]. The presence of a stripy geom-

etry, for example, stimulates the orientation of cells and alters their morphology, sometimes into a very extended form; for this reason studying cell movement appears to be the most promising approach. Some kinds of cells, such as fibroblasts and endothelial cells, tend to fall into line on the concave side of stripes hollowed out on silicon surfaces [7]. In particular, it has been shown that the primary fibroblast orientation increases when in contact with stripes 10-30 nm in size [8]. It is not yet clear whether this alignment in the grooves is due to a particular conformation assumed by the cell when in contact with the groove or to other events, such as medium accumulation in these particular geometries. The contact of macrophages with a particular topography causes some change in the cytoskeleton in short times, and stimulates cellular phagocytosis. All this suggests that cell behavior depends on the cells investigated.

Structures with chemical stimulus can also be realized. For example, by alternating on the same surface adhesive nanometric domains for cells, such as those made of proteins like fibronectin and vitronectin, with nonadhesive domains, such as polymers like polyethylene glycol, cells can be forced to adhere, migrate and proliferate only on the adhesive areas. Plasma enhanced chemical vapor deposition (PE-CVD) and plasma treatments (surface modification for functional group grafting) are used for this purpose [9]. Figure 4.1 shows an example of fibroblast adhesion on polyethylene terephthalate (PET) substrates treated with the plasma-deposition process to obtain nanostructured fluorocarbon coatings. Rosso et al. [10] showed that both cell morphology and internal signal transduction pathways of the adhering fibroblasts on rough surfaces were quite different compared to cells adhering on smooth ones.

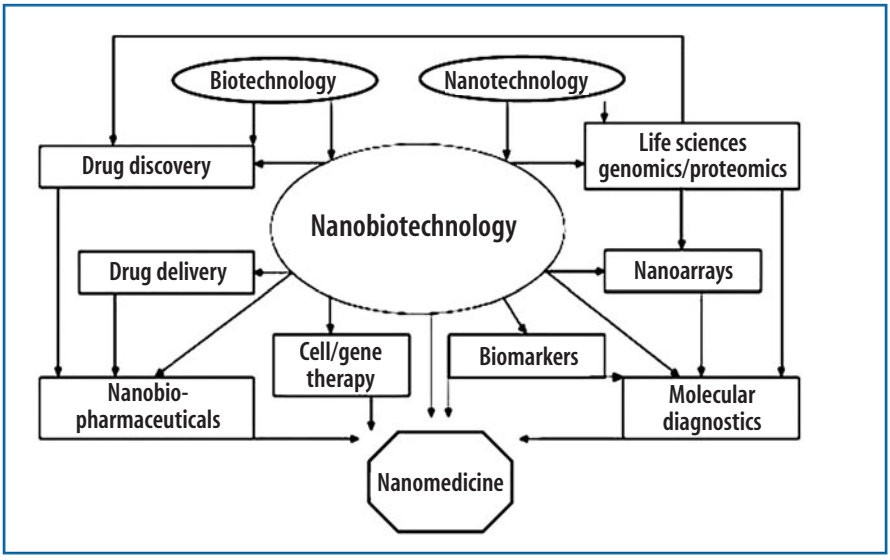

Fig. 4.1 Interrelations between nanomedicine and nanobio-technology

Metals with nanostructured surfaces can be used in orthopedics and the dental field. Mechanical and chemical characteristics make metals (Ti, etc.) fit to play a *supporting* role, while the presence of nanostructures on the surface makes the prosthesis suitable for favoring the process of biointegration.

Other studies have been devoted to the preparation of organic/inorganic hybrid materials, with particular chemical and morphological characteristics, aiming to control or favor biomineralisation processes of hard tissues [11]. The realization of organic/inorganic hybrid materials means developing new nanostructured materials which can be used as hard tissue fillings or orthopedic and dental prosthesis coatings.

The application of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems is now often referred to as nanomedicine: the complex interrelations between nanomedicine and nanobiotechnology are shown in Figure 4.2.

Among many possible applications of nanotechnology in medicine, the use of various nanomaterials as pharmaceutical delivery systems for drugs, DNA, and imaging agents has gained increasing attention. Many varieties of nanoparticles are available [1], such as different polymeric and metal nanoparticles, liposomes, niosomes, solid lipid particles, micelles, quantum dots, dendrimers, microcapsules, cells, cell ghosts, lipoproteins, and different nanoassemblies.

The paradigm of using nanoparticulate pharmaceutical carriers to enhance the in vivo efficiency of many drugs, anti-cancer drugs first of all, has become well established over the past decade, in both pharmaceutical research and the clinical setting. Numerous nanoparticle-based drug delivery and drug targeting systems have already been developed or are under development [12]. Their use aims to minimize drug degradation and inactivation upon administration, prevent undesirable side effects, and increase drug bioavailability and the fraction of drug delivered to the pathological area. In general, pharmaceutical drug carriers, especially those used for parenteral administration, are expected to be biodegradable, easy and reasonably

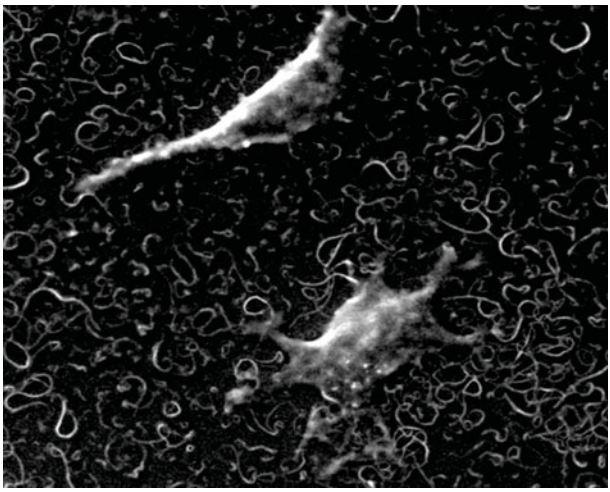

Fig. 4.2 Environmental scanning electron microscopy (ESEM) image of fibroblast adhesion on fluorocarbon nanostructured surface

cheap to prepare, to have a small particle size, to possess a high loading capacity, to demonstrate prolonged circulation, and ideally to accumulate specifically or non specifically in required sites in the body.

Some time ago, it was found that high-molecular-weight long-circulating macromolecules as well as various long-circulating nanoparticulate pharmaceutical carriers are capable of spontaneous accumulations in various pathological sites, such as solid tumors and infarcted areas, via the so-called enhanced permeability and retention (EPR) effect [13]. This effect is based on the fact that pathologic vasculature, unlike vasculature of normal healthy tissues, is *leaky* – that is, penetrable for large molecules and even for small particles – which allows for their extravasation and accumulation in an interstitial tumor space. Such accumulation is additionally facilitated by the virtual lack in many tumors of a lymphatic system, responsible for the drainage of macromolecules from normal tissues. It has been found that the effective pore size in the endothelial lining of the blood vessels in most peripheral human tumors ranges from 200 nm to 600 nm in diameter, and the EPR effect allows for passive targeting of tumors based on the cutoff size of the leaky vasculature.

The most extensively studied particulate drug carriers are liposomes, micelles, and polymeric nanoparticles, which possess the most suitable characteristics for the encapsulation of many drugs, genes, and diagnostic (imaging) agents. These drug carriers as well as any other pharmaceutical nanocarriers can be surface modified by a variety of different moieties to impart them with certain properties and functionalities. These functionalities are expected to provide nanocarriers with:

1. prolonged circulation in the blood and ability to accumulate in various pathologic areas (e.g. solid tumors) via the EPR effect (protective polymeric coating with polyethylene glycol [PEG] is frequently used for this purpose);
2. the ability to specifically recognize and bind target tissues or cells via the surface-attached specific ligands such as folate and thiamine, peptides such as RGD, and sequences identified by phage display, proteins such as transferrin, lectins, antibodies and antibody fragments, polysaccharides such as galactose, and aptamers;
3. the ability to respond to local stimuli characteristic of the pathologic site by, for example, releasing an entrapped drug or specifically acting on cellular membranes under the abnormal pH or temperature in disease sites (this property could be provided by surface-attached pH-sensitive or temperature-sensitive components);
4. the ability to penetrate inside cells bypassing lysosomal degradation for efficient targeting of intracellular drug targets (for this purpose, the surface of nanocarriers is additionally modified by cell-penetrating peptides).

These are just the most evident examples. Figure 4.3 shows fibroblast uptake of hyaluronic acid nanoparticle loaded with fluorescent rhodamine. Some other specific properties can also be listed, such as an attachment of diagnostic moieties. Even the use of individual functionalities is already associated with highly positive clinical outcome; the success of Doxil, doxorubicin in a long-circulating PEG-coated liposome, is a good example [14].

Patient research has shown the impressive effects of doxorubicin in PEG lipo-

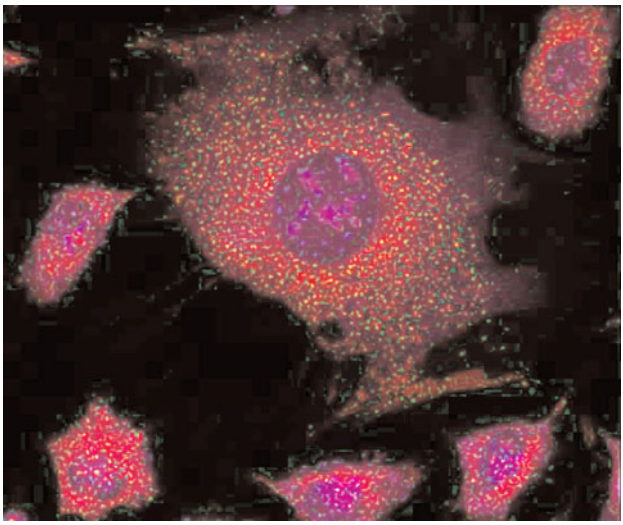

Fig. 4.3 Fibroblast uptake of hyaluronic acid nanoparticle loaded with fluorescent rhodamine. Image from laser confocal microscopy

somes against metastatic breast carcinoma, unresectable hepatocellular carcinoma, cutaneous T-cell lymphoma, sarcoma, squamous cell cancer of the head and neck, and ovarian cancer [15].

4.2 Biosensors

Biosensors are versatile analytical tools used in many fields such as clinical diagnostics, detection of industrial pollution, control of fermentation and also in the field of security and safety for the monitoring of both chemical and biological hazardous agents.

The main requirements for a biosensor approach to be valuable in terms of research and commercial applications are the identification of a target molecule, availability of a suitable biological recognition element, and the potential for disposable portable detection systems to be preferred to sensitive laboratory-based techniques in some situations. Some examples are given below:

- glucose monitoring in diabetes patients;
- other medical health related targets;
- environmental applications e.g. the detection of pesticides and river water contaminants;
- remote sensing of airborne bacteria e.g. in counter-bioterrorist activities;
- detection of pathogens [16];
- determining levels of toxic substances before and after bioremediation;
- detection and determining of organophosphate;

- routine analytical measurement of folic acid, biotin, vitamin b12 and pantothenic acid as an alternative to microbiological assay;
- determination of drug residues in food, such as antibiotics and growth promoters;
- drug discovery and evaluation of biological activity of new compounds;
- detection of toxic metabolites such as mycotoxins [17].

A biosensor is a device for the detection of an analyte (a chemical compound that you want detect) that combines a biological component with a physicochemical detector component. It consists of three parts:

- the *sensitive biological element;* a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc), a biologically derived material or biomimetic, or sensitive elements created by biological engineering;
- the *transducer* or the *detector element* (works in a physicochemical way; optical, piezoelectric, electrochemical, etc.) that transforms the signal resulting from the interaction of the analyte with the biological element into another signal (i.e. transducers) that can be more easily measured and quantified;
- associated electronics or signal processors that are primarily responsible for the display of the results in a user-friendly way [18].

Hence, a biosensor uses biologically active molecules called *probe* (enzymes, receptors, proteins, nucleic acids) or molecules that can recognize with high specificity an analyte present in a complex matrix. The biological response that comes from probe-analyte interaction (conformational changes of biomolecules) is converted into a quantifiable electrical, optical or chemical signal which is amplified and then converted by a microprocessor in concentration units. Furthermore, a biosensor has another important component which is the microfluidic device, with micro-pump for the transport of the solution that will be analyzed. Moreover, the use of enzymes and proteins as biosensors is largely supported by the progress of genetic engineering that allows not only the production of large amounts of protein but also the modification of its properties.

An important part in a biosensor is the attachment of the probes (small molecules/protein/cells) to the surface of the sensor (be it metal, polymer or glass). The simplest way is to chemically functionalize the surface in order to coat it with the biological elements. This can be done by polylysine, aminosilane, epoxysilane or nitrocellulose in the case of silicon chips/silica glass. Subsequently the bound biological agent may be for example fixed by layer-by-layer deposition of alternatively charged polymer coatings [19].

Alternatively, the probe can be immobilized on materials such as graphite, gold, silicon crystal fixed to the glass, or polymer-porous silicon (PSi), which is the most innovative and most widely used for its high biocompatibility. The porous silicon due to its characteristic sponge-like morphology is a nearly ideal material to use as a transducer optical biosensors. Its inner surface in fact, has a specific area that is of the order of 200-500 m^2/cm^3 so that the interaction with the adsorbed substances, liquid or gas is very effective.

The porous silicon is produced by electrochemical attack of a silicon crystal

wafer. When the reaction is complete, the entire surface of porous silicon appears covered with Si-H bonds, which are thermodynamically unstable. These are then replaced with Si-C bonds, which are very stable and resistant to chemical attack. This makes it possible to functionalize the porous silicon with enzymes, antibodies, proteins or DNA sequences: for example, the silicon group will react with a carboxylic acid amide group of a lysine, thus obtaining a protein covalently linked to the surface of the biosensor. To test the protein bond on the silicon wafer, polypeptides are labeled with a fluorophore (e.g. rhodamine) and if after a series of extensive washes the fluorescence persists, the reaction has been successful.

Physical probe trapping is achieved using three dimensional lattices (hydrogel). The most commonly used hydrogel is a glassy silica generated by the polymerization of silicate monomers in the presence of the biological elements (along with other stabilizing polymers, such as polyethylene glycol) [18]. Another group of hydrogels, which set under conditions suitable for cells or protein, are acrylate hydrogel, which polymerizes upon radical initiation [20].

Regardless of the probe immobilization method on silicon wafers, the partial replacement of the air inside the pores with the biomolecule and/or the biomolecule-ligand complex corresponds to a gradual increase in the average refractive index of porous silicon. This increase results in a shift toward longer wavelengths (red-shift) of the reflection spectrum, which is obtained by hitting the porous silicon with a beam of collimated light.

Several methods have been used for the realization of stable and specific biosensors. Fluorescence spectroscopy, due to high sensitivity, low cost and ease of use, is the *gold standard* in biotechnology.

The stability properties of these biosensors are primarily derived from the use of thermostable proteins and enzymes isolated from extremophile organisms, which constitute a rich source of molecules with unique specificity and stability even under extreme conditions of temperature, pH and salinity or in the presence of detergents and organic solvents. The enzymes isolated from extremophiles show a higher stability than their mesophilic counterparts, even in conditions considered optimal for mesophilic organisms.

Protein biosensors have been developed in the biomedical field for the quantitative determination of glucose using the glucose binding protein (GBP) as a probe.

At present the use of enzymes (e.g. alcohol dehydrogenase) as a biosensor has the disadvantage of consuming the substrate, this represents a real problem in the design of implantable biosensors, that besides being precise and minimally invasive instruments allow a continuous monitoring (night and day) of analytes such as ethanol in the blood.

Recently, it was demonstrated that apo-enzymes (enzymes without coenzyme) can be used as probes for the development of fluorescent biosensors. Apo-enzymes, antibodies and proteins belonging to the family of receptor-binding protein can then bind the substrate without turning it into product. This biosensor is not only accurate but also stable and reversible. The biomolecule-substrate interaction triggers small conformational changes that can be easily identified through fluorescence measurements and/or polarization.

Recently, arrays of many different detector molecules have been applied in so-called electronic nose devices, where the pattern of response from the detectors is used to fingerprint a substance. Current commercial electronic noses, however, do not use biological elements.

4.3 Nanodiagnostics

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Nanodiagnostics can be defined as the use of nanotechnology for clinical diagnostic purposes. The increased demand for sensitivity requires that a diagnostically significant interaction occurs between analyte molecules and signal-generating particles, thus enabling detection of a single analyte molecule. Nanotechnology has enabled one-to-one interaction between analytes and signal-generating particles such as quantum dots (QDs; 2-8 nm) in the size range of proteins (1-20 nm) and other important biomolecules [1].

Nanotechnology implemented within current diagnostic equipment has the potential of analyzing entire genomes in minutes instead of hours. Based on which DNA sequences are deviated from the normal, we will be able to determine an individual's predisposition to either cancer or a specific disease.

A recent development is the *nanochip*, which employs the power of an electronic current that separates DNA probes to specific sites on the array based on charge and size on specific sites of the nanochip. The test sample (blood) can then be analyzed for target DNA sequences by hybridization with these probes. The DNA molecules that hybridize with target DNA sequences fluoresce, which is detected and relayed back to an onboard system through platinum wiring that is present within the chip. The secret behind this nanochip is that each test site can be controlled electronically from the system's onboard computer. In other words, the chip can place different probes in different sites according to what DNA sequence is of interest. Therefore, if a mutation in the DNA sequence of a gene that causes a disease is known, we will be able to know whether or not a person is predisposed to that particular disease if hybridization with that probe occurs.

The newest technologies within nanodiagnostics involve microfluidic or *lab-on-a-chip* systems. The idea behind this kind of chip is simple: the combination of numerous processes of DNA analysis are combined on a single chip composed of a single glass and silicon substrate. The device itself is composed of microfabricated fluidic channels, heaters, temperature sensors, electrophoretic chambers, and fluorescence detectors to analyze nanoliter-size DNA samples.

Related to microfluidic systems are microelectromechanical systems or MEMS. The difference between MEMS and microfluidic systems are that MEMS do not require reagents or a fluidity based substrate to react upon. Although MEMS are primarily used in drug-delivery systems, one primary application of MEMS in diagnostics are the swallowed capsule technology pills that allow doctors to visualize internal bleedings.

Practically, the patient swallows a capsule containing a light emitting diode for illumination, a CMOS (complementary metal-oxide semiconductor) video camera and optics for taking images, a battery, and a transmitter [21]. The images are then transmitted to a receiver worn on the patient's belt and the doctor is then able to diagnose the cause of the ailment.

In recent years, studies on nanoparticles have given a considerable impetus to nanodiagnostics. Nanoparticles possess certain size-dependent properties, particularly with respect to optical and magnetic parameters, which can be manipulated to achieve a detectable signal. The primary event in most nanoparticle-based assays is the binding of a nanoparticle label or probe to the target biomolecule that will produce a measurable signal characteristic of the target biomolecules. A variety of probes have been used for this purpose, including QDs, cantilevers, nanoshells, and metal nanoparticles [22].

QDs are the most used and promising nanostructures for diagnostic applications. QDs are semiconductor nanocrystals, with a diameter of 2-8 nm, characterized by strong light absorbance, that can be used as fluorescent labels for biomolecules. QDs can be conjugated to antibodies, an oligonucleotide or aptamer, or coated with streptavidin. This helps direct the QD toward the target analyte. In addition, QDs can be used as nonspecific fluorescent labels.

When a QD absorbs a photon with energy higher than the band-gap energy of the composing semiconductor, an exciton, or electron-hole pair, is created. As a result, a broadband absorption spectrum occurs because of the increased probability of absorption at shorter wavelengths. The return of the exciton, characterized by a long lifetime >10 ns, to a lower energy state leads to emission of a photon with a narrow symmetric energy band [23]. QDs are reported to emit with lifetimes of 5-40 ns, whereas conventional organic dyes emit in the 0.5-2 ns range. This produces a strong, stable fluorescence signal.

Methods for tracking and detecting QDs are numerous and include fluorometry and several types of microscopy, such as fluorescence, confocal, total internal reflection, wide-field epifluorescence, atomic force, and multiphoton microscopy [22]. The choice of monitoring or detection technique depends on the type of application, such as in vitro diagnostics or imaging, for which QDs are used.

The advantages of QD over conventional organic dyes and fluorophores is their optical tunability, resistance to photobleaching, excitation of various QDs by a single wavelength of light (for multiplexing), narrow emission band, and exceptional stability of optical properties after conjugation to a biomolecule. Currently, up to 40,000 assays can be run simultaneously with the current capabilities of the QD technology [1].

The use of QD labels for DNA detection would help overcome two of the most important problems encountered when using organic dyes for DNA labeling; cleavage of DNA molecules as a result of photobleaching and subsequent formation of free radicals, and disturbing DNA–protein interactions.

QDs are at the core of a fluoroimmunoassay introduced by Härmä et al. [24] for the detection of prostate-specific antigen (PSA). QDs have also recently been used to optically detect acetylcholine [25]. Peptides have also been conjugated to QDs, as

exemplified by the work of Akerman et al. [26]. These investigators conjugated three different peptides to QDs to target lung endothelial cells, brain endothelial cells, and breast carcinoma cells, both in vitro and in vivo. Microinjection of QDs conjugated to suitable peptides can also target cellular organelles such as the nucleus or mitochondria.

Another promising area for application of QDs is the detection of cancer. An assay has been developed for detection of the receptor Her2 (hairy-related 2) on SKBR-3 breast cancer cells [27].

QDs have applications in cellular imaging. Antibody-coupled QDs injected into the tail veins of mice were successful in detecting prostate cancer xenografts. The spectral image of the mice clearly showed the location of the target-bound QDs [23]. A more generalized imaging application was described by Larson et al. [28], who injected water-soluble QDs into mice to image skin and adipose tissues. Despite the fact that both of these tissues have high light-scattering properties, the vasculatures containing QDs at about 1 μmol/L were clearly visible by multiphoton microscopy.

The most eminent problem regarding QDs is toxicity. The fact that QDs have basic components that are highly toxic to humans, e.g. cadmium, raises serious safety issues. Many of the studies mentioned previously used QDs with concentrations just high enough to achieve optimal labeling, and the duration of use ranged from several hours to several days. The authors of these studies found no adverse effects on cellular functioning or development during the study. However, at higher QD concentrations (>5 x10^9 QDs/cell), *Xenopus* embryo development was affected [23]. For in vivo studies, the question that remains is whether the surface coating, assuming one is used, of the QD will be sufficiently robust to guard against leakage of the core metals and semiconductors into the biological system being studied.

The same factors that lead to questions concerning the safety of QDs and other nanostructures for use in humans lead to questions of environmental safety. Specifically, can QDs be stored and disposed of without leakage of the toxic metals used?

Gold nanoparticles and gold nanoshells provide great sensitivity for the detection of DNA and proteins. They can be used to label DNA or protein molecules (including antibodies), which can then bind to their respective targets [1].

The optical technique called *surface plasmon resonance* measures the optical change produced from the interaction of locally adjacent gold nanoparticle (bounded to a specific target) and then uses this optical change for target detection [29].

The bio-barcode assay (BCA) has been used for the detection of proteins such as PSA, which is an important marker for prostate and breast cancers [30]. BCA has also been used to successfully detect a biomarker for Alzheimer disease. In a study by Georganopoulou et al. [31], the BCA was used to determine the concentration of the biomarker, amyloid beta-derived diffusible ligands (ADDLs), in cerebrospinal fluid.

The assay, which is one million-fold more sensitive than the current ELISA technique, could be developed for the measurement of ADDLs in blood, thus providing an early diagnostic tool for Alzheimer disease.

The promise of increased sensitivity and speed and reduced cost and labor makes nanodiagnostics an appealing alternative to current diagnostic techniques. The potential diagnostic uses of QDs are numerous, with the most promising applications being in the areas of tumor detection, tissue imaging, intracellular imaging, immunohistochemistry, infectious agent detection, multiplexed diagnostics, and fluoroimmunoassays.

QDs also have considerable potential for in vivo imaging, but there are concerns over their toxicity, both to patients and the environment. The technologies are numerous and the applications are constantly increasing, with QDs, gold nanoparticles, and BCAs in the lead. The numerous types of nanoparticles differ in shape and properties, which could be used for specific diagnostic applications.

PCR could soon lose its lead position as the gold standard for DNA detection to BCA, which offers increased sensitivity and safety. In time, nanodiagnostics may become very cost-effective, as is currently the case with some magnetic nanoparticles. This should allow better clinical diagnostic services, particularly in economically deprived regions. These technologies can also be applied to point-of-care testing and lab-on-a-chip technologies. Whether nanodiagnostics will replace current diagnostic methods remains to be seen. Many aspects of these nanodiagnostic techniques need to be evaluated further, especially the safety issues.

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Regenerative Medicine: Current and Potential Applications

5

A. Barbarisi, F. Rosso

5.1 Scaffold and Molecular Signals for Tissue Engineering

There is a substantial unmet demand for tissues to repair injured, degenerated or congenitally defected tissues. The field of tissue engineering has emerged to fill the void where neither native physiology nor purely artificial implantable materials can sufficiently replace or repair these damaged tissues. While tissues such as bone or skin can effectively repair a small injury given sufficient time, many tissues such as myocardium and cartilage do not regenerate properly without intervention.

To achieve this, it is necessary to combine the use of cells together with natural or synthetic scaffolds in or onto which cells can develop, organize and behave as if they are in their native tissue. Therefore, cells must receive signals from the environment to carry out in an orderly way proliferation and differentiation programs aimed at tissue/organ formation [1].

With regard to these biological process cells need a continuous flow of signals from the surrounding extracellular environment that allow for specific genetic program fulfillment. It is tempting to generalize that in all tissues cells live in contact with matrices or scaffolds. From the early developmental phases onwards, embryonic cells produce their own extracellular scaffolds by secreting many types of molecules in the surrounding space, according to a well defined program of differentiation [2]. The different spatial organization of these secreted molecules gives rise to a great variety of natural scaffolds in which cells continue to proliferate and to organize themselves to build tissues and to accomplish all their natural functions. The understanding of cell differentiation and functions means the under-

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standing of cell–cell and cell–extracellular matrix (ECM) communication mechanisms. In this respect, if one looks considering the complexity of tissue ECM components, it is not surprising to find the same great variety and complexity of existing interactions between cells and ECM. Bearing these basic considerations in mind, our efforts in trying in vitro tissue reconstruction must be driven toward the exact knowledge of cell function on the one hand, and on the other toward the knowledge of interactions and signals that cells must receive from the environment to behave as in natural tissues [1].

Tissue-engineering approaches typically employ exogenous three-dimensional ECMs to engineer new natural tissues from natural cells. The exogenous ECMs are designed to bring the desired cell types into contact in an appropriate three dimensional environment, and also to provide mechanical support until the newly formed tissues are structurally stabilized and specific signals occur to guide the gene expression of cells forming the tissue [3].

All synthetic ECMs used to engineer tissues have three primary roles. First, the synthetic ECMs facilitate the localization and delivery of cells to specific sites in the body. Second, they define and maintain a three dimensional space for the formation of new tissues with the appropriate structure. Third, they guide the development of new tissues with their appropriate functions.

The synthetic ECM should provide temporary mechanical support sufficient to withstand in vivo forces and maintain a potential space for tissue development. This mechanical support by the synthetic ECM should be maintained until the engineered tissue has sufficient mechanical integrity to support itself, as in the case of the Kim and Mooney [4] studies that showed the ability of synthetic ECMs based on nonwoven mesh of polyglycolic acid bonded at their fiber cross points with poly-L-lactic acid to resist cellular contractile forces and maintain their predefined structure during the process of smooth muscle tissue development in vitro.

The cells composing the engineered tissue must express the appropriate genes to maintain the tissue specific function of the engineered tissue. The function of seeded cells is strongly dependent on the specific cell surface receptor (e.g. integrins) used by the cell to interact with the material, on interactions with surrounding cells, and on the presence of soluble growth factors. These factors can be controlled by incorporating or integrating a variety of signals, such as cell-adhesion peptides and growth factors [5] into the synthetic ECM or by subjecting it to mechanical stimuli [6].

The exogenous ECMs for tissue engineering can be fabricated from two classes of biomaterials: naturally derived materials and synthetic materials. Naturally occurring materials are composed of polypeptides, polysaccharides, nucleic acids, hydroxyapatites, or their composites. Biological materials have some remarkable advantages over synthetics, that is, their excellent physiological activities such as selective cell adhesion (e.g. collagen and fibrin), mechanical properties similar to natural tissues (e.g. animal heart valves and blood vessels), and biodegradability (e.g. gelatine and chitin). However, as with synthetics, biological materials have several deficiencies including risk of viral infection, antigenicity, unstable material supply and the deterioration which accompanies long-term implantation. In addition, naturally derived materials offer limited versatility in designing an exogenous

ECM with specific properties (e.g. porosity, mechanical strength). Synthetic materials, by contrast, can be manufactured reproducibly on a large scale, and can also be processed into an exogenous ECM in which the macrostructure, mechanical properties, and degradation time can be readily controlled and manipulated. Exogenous ECMs fabricated by biodegradable polymers will eventually erode in the body, avoiding a chronic foreign-body response.

The greatest disadvantage of synthetic materials, however, is the lack of cell-recognition signals. Toward this end, efforts are being made to incorporate cell adhesion peptides into biomaterials, which normally exhibit few cellular interactions. Mechanical signals conveyed to cells via their adhesion to the matrix also clearly regulate the development of various tissues and the gene expression of many cell types in culture. The concept of combining synthetic materials with cell-recognition sites of naturally derived biomaterials is very attractive. These hybrid materials could possess the favorable properties of synthetic materials, including widely varying mechanical and degradative properties, reproducible large-scale production, and good processability, as well as the specific biological activity of naturally derived materials. This latter property may be needed to engineer complex tissue with multiple cell types organized in specific patterns. Several cell-adhesion ligands with highly specific recognition could potentially be displayed spatially in a desirable pattern to induce specific cell-organization schemes [7].

Many researchers have been focusing on the development of semi-synthetic materials that are targeted to assist tissue regeneration [8, 9]. Placed at the site of a defect, such materials should actively and temporarily participate in the regeneration process by providing a platform on which cell-triggered remodeling could occur.

In particular, the ability to supply molecular signals able to guide the cellular response on these new semi-synthetic biomaterials represent a tremendous innovation in the area of tissue engineering.

5.2 Cell Source for Tissue Engineering

The choice of cells for creation of body structures is a major challenge in tissue engineering. The main goal in this regard – the large-scale fabrication of structures – may be to create large cell banks composed of universal cells that would be immunologically transparent to an individual. These universal cells could be differentiated cell types that could be accepted by an individual or could be stem cells, which could respond to signals to differentiate into differing lineages for specific structural applications. Our knowledge about the biology of stem cells and their existence in various mesenchymal tissues (muscle, bone, and cartilage), endodermally derived tissues (intestine and liver), or ectodermally derived tissues (nerves, pancreas, and skin) expands on a daily basis. A new area of stem cell biology involving embryonic stem cells holds promise for tissue engineering. The development of

immunologically inert universal cells may come from advances in genetic manipulation as well as stem cell biology. As intermediate steps, tissue can be harvested as allograft, autograft, or xenograft. The tissues can then be dissociated and placed into cell culture, where proliferation of cells can be initiated. After expansion to the appropriate cell number, the cells can then be transferred to templates, where further remodeling can occur. Which of these strategies are practical and possibly applicable in humans remains to be explored. Large masses of cells for tissue engineering need to be kept alive, not only in vitro but also in vivo. Moreover, not only will one need to have a sufficient supply and one that can be ensured to be free of pathogens and contamination, but one will need to decide whether the source to be employed is to be autologous, allogeneic, or xenogeneic. With regard to the use of autologous cells, there are a number of potential sources. These include both differentiated cells and adult stem/progenitor cells. For some tissue-engineering applications currently under development the ability to obtain a tissue biopsy and expand a sufficient number of autologous cells is well established. In other circumstances it is not clear how a patient's own cells could be harvested and/or expanded to yield enough material for production of the needed neo-tissue or organ. Cardiomyocytes, neurons of the central nervous system, hepatocytes and other liver cells, kidney cells, osteoblasts, and insulin producing pancreatic beta-cells are examples of differentiated cell types for which new sources could enable novel therapies to address significant unmet medical needs.

Immature precursor cells present within tissue samples are essential for the expansion of cells from biopsies of skin, bladder, or cartilage that enables the engineering of the corresponding neo-tissues. The ability to extend tissue engineering to other tissue and organ systems will depend greatly on finding sources of appropriate stem and progenitor cells. Three major sources currently are under intensive investigation: (1) embryonic stem (ES) and embryonic germ (EG) cells derived from discarded human embryos and germ line stem cells, respectively; (2) ES cells created by somatic cell nuclear transfer (therapeutic cloning); and (3) *adult* stem cells from fetal, neonatal, or adult tissue, either autologous or allogeneic.

Hence, there is a variety of different stem cells. It is the adult stem cells and progenitor cells that are being and will be used first clinically; however, in the long term there is considerable interest in embryonic stem cells. These cells are pluripotent, i.e. capable of differentiating into many cell types, even totipotent, i.e. capable of developing into all cell types. Although we are quite a long way from being able to use embryonic stem cells, a number of companies are working with stem cells in the context of tissue engineering and regenerative medicine. It needs to be recognized, however, that immunogenicity issues may be associated with the use of embryonic stem cells. Furthermore, different embryonic stem cell lines, even when in a totally undifferentiated state, can be significantly different. This is illustrated by the results of Rao et al. [10] in a comparison of the transcriptional profile of two different embryonic stem cell lines. This difference should not be considered surprising, since the lines were derived from different embryos and undoubtedly cultured under different conditions. To take full advantage of stem cell technology, however, it will be necessary to understand more fully how a stem cell differentiates

into a tissue-specific cell. This requires knowledge not just about the molecular pathways of differentiation, but, even more importantly, about the identification of the combination of signals leading to a stem cell's becoming a specific type of differentiated tissue cell. In addition, however, we will need to develop the technologies necessary to expand a cell population to the number necessary for clinical application, be able to do this in a controlled, reproducible manner, and deliver cells at the right place and at the right time.

The ES cells have a great degree of plasticity, which is the most attractive feature of the use of these cells for regenerative medicine. A major remaining challenge is to direct the efficient production of pure populations of specific desired cell types from human ES cells. In fact, undifferentiated ES cells of murine and human origin form teratomas in vivo containing an array of cell types, including representatives of all three embryonic germ layers [11, 12]. Therefore, it will be important to document rigorously the exclusion of undifferentiated stem cells from any tissue-engineered products derived from ES cells [13]. Strategies have been envisaged to increase safety by introducing into ES cells a suicide gene, which would render any escaping tumor cells sensitive to the drug ganciclovir [14]. However, the genetic manipulation is itself not without risk, and the need to validate the engineered cell system would likely extend and complicate regulatory review of therapeutic products.

A central issue that must be addressed for tissue engineered products derived from ES cells, and also from any nonautologous adult stem cells, is immune rejection based on mismatches at genetic histocompatibility loci [15]. It generally has been assumed that, because human ES cells and their differentiated derivatives can be induced to express high levels of MHC Class I antigens (e.g. HLA-A and HLA-B), any ES cell-based product will be subject to graft rejection [16].

Therapeutic cloning offers a potential means to generate cells with the exact genetic constitution of each individual patient so that immune rejection of grafts based on mismatched histocompatibility antigens should not occur.

The approach entails transferring the nucleus of a somatic cell into an enucleated oocyte (SCNT), generating a blastocyst, and then culturing the inner cell mass to obtain an ES cell line [17].

If required, genetic manipulation of the cells may be carried out to correct an inherited defect prior to production of the therapeutic graft. Adult bovine fibroblasts were used as nuclear donors and bioengineered tissues were generated from cloned cardiac, skeletal muscle, and kidney cells [18]. The grafts, including functioning renal units capable of urine production, were successfully transplanted into the corresponding donor animals long term, with no evidence of rejection. Although SCNT technology is the subject of study by both private and public research, it is still the focus of political, ethical, and scientific debate.

The clinical application of ES cells for tissue engineering will depend on the development of robust methods to isolate and grow them under conditions consistent with good manufacturing practice and regulatory review for safety. In particular, it is important to eliminate the requirement for murine feeder cells by using human feeders or, better, feeder free conditions. In addition, development of culture

conditions without the requirement for nonhuman serum would be advantageous. Progress has been made in the derivation and expansion of human ES cells with human feeder cells [19, 20] or entirely without feeders [21, 22].

Perhaps the greatest challenge remains in directing the differentiation of human ES cells to a given desired lineage with high efficiency. To induce differentiation in vitro, ES cells are allowed to attach to plastic in monolayer culture or, more frequently, to form aggregates called embryoid bodies [23]. Over time within these aggregates cell types of many lineages are generated, including representatives of the three germ layers. The production of embryoid bodies can be enhanced and made more consistent by incubation in bioreactors [24]. Further selection of specific lineages generally requires sequential exposure to a series of inducing conditions based on known signaling pathways. As summarized in recent reviews, the cell lineages that have been generated in vitro include, among others, several classes of neurons, astrocytes, oligodendrocytes, multipotent mesenchymal precursor cells, osteoblasts, cardiomyocytes, keratinocytes, pneumocytes, hematopoietic cells, hepatocytes, and pancreatic beta-cells [25-27]. In general, it appears easier to obtain adult cells derived from ectoderm, including neurons, and mesoderm, including cardiomyocytes, than cells derived from endoderm. This may help determine the earliest areas in which ES-derived cells enter clinical translation, once the barriers just discussed are surmounted. Dopaminergic neurons generated from primate and human ES cells already have been tested in animal models of Parkinson's disease, with encouraging results [28]. Cardiomyocytes derived from human ES cells, similarly, are candidates for future clinical use, although the functional criteria that must be met to ensure physiological competence will be stringent because of the risk of inducing arrhythmias [29, 30].

In many cases adult stem cells may provide a more direct route to clinical translation. Lineage-restricted stem cells have been isolated from both fetal and postnatal tissues based on selective outgrowth in culture and/or immunoselection for surface markers.

Their ability to expand may be less than that for ES cells, as they are multipotent not pluripotent. Neural stem cells can yield neurons, astrocytes, and oligodendrocytes. Cardiac stem cells are reported to yield cardiomyocytes, smooth muscle, and endothelial cells. Muscle-derived stem cells yield skeletal muscle and can be induced to produce chondrocytes. Hepatic stem cells yield hepatocytes and bile duct epithelial cells. The lineage restricted adult stem cells all appear non tumorigenic. Thus, unlike ES cells, it is likely that they could be used safely for bioengineered products with or without prior differentiation.

It is possible that some lineage-specific adult stem cells are capable of greater plasticity than might be supposed based solely on their tissue of origin. For example, there is evidence that mesenchymal stem cells (MSC), initially described in bone marrow, are able to give rise to differentiated cells of connective tissues, including bone, cartilage, muscle, tendon, and fat. The MSC have therefore generated considerable interest for musculoskeletal and vascular tissue engineering [31, 32]. Cells with similar differentiation potential and marker profiles have been isolated from a number of tissues in addition to the bone marrow. A notable source is

adipose tissue, in which the cells are abundant and easily obtained by processing of suction-assisted lipectomy (liposuction) specimens [33, 34].

In general it seems better to view MSC as mixed populations of progenitor cells with varying degrees of replicative potential, rather than homogeneous stem cells. Cells originating in a developing fetus and isolated from amniotic fluid or chorionic villi are a new source of stem cells of great potential interest for regenerative medicine [35]. Fetal-derived cells with apparently similar properties also have been described in the amnion of term placenta [36]. Amniotic fluid stem (AFS) cells and amniotic epithelial cells can give rise to differentiated cell types representing the three embryonic germ layers [35]. Formal proof that single AFS cells can yield this full range of progeny cells was obtained using clones marked by retroviral insertion. The cells can be expanded for well over 200 population doublings, with no sign of telomere shortening or replicative senescence, and retain a normal diploid karyotype. They are readily cultured without need for feeder cells. A broadly multipotent cell population obtained from umbilical cord blood may have certain key properties in common with AFS cells, and it was termed *unrestricted somatic stem cells* (USSCs) [37].

Similar approaches to those being taken with ES cells, such as genetic modification with expression vectors for lineage specific transcription factors, may help in the generation of differentiated cell types for which it proves difficult to develop a straightforward induction protocol using external signals.

Keeping in mind that the only cell based therapies guaranteed to be histocompatible would contain autologous cells, when we use other cell sources, the question of immune compatibility comes up.

There are many experimental data showing the immune privilege of stem cells. In fact, human ES cells express low levels of class I major histocompatibility complex (MHC) antigens (HLA-A, HLA-B) and are negative for MHC class II [16].

Differentiated derivatives of the ES cells remain negative for MHC II but show some increase in MHC class I that is up-regulated by exposure to interferon. These observations gave rise to the natural assumption that ES cells and their differentiated progeny would be subject to rejection based on MHC mismatches and led to a search for strategies to induce immunological tolerance in recipients of transplanted cells derived from ES lines.

However, it was observed that ES cells in the mouse could be transplanted successfully in immunocompetent animals despite mismatches at the major histocompatibility loci [38, 39]. Even more remarkably, human ES cells and differentiated derivatives were not rejected by immune-competent mice in vivo, nor did they stimulate an immune response in vitro by human T lymphocytes specific for mismatched MHC.

An independent study using mice with a *humanized* immune system confirmed a very low T-cell response to human ES cells and differentiated derivatives [40].

The successful therapeutic use of allogeneic MSC has been confirmed in animal models [41]. Beyond the use of MSC as regenerative cells, it is possible that they could be employed to induce immune tolerance to grafts of other cell types. The mechanisms underlying the immunomodulatory properties of MSC are under active

investigation, and understanding them may have a profound impact on regenerative medicine [42, 43]. Taken together these data support the idea that allogeneic bioengineered products may not require intensive immunosuppressive treatment.

Finally, for all tissue engineered products, a critical issue will be how they are delivered and made available to the clinician. In other words there is a need to develop an appropriate product quantity and a cost-effective manufacturing process. A lot of the research on manufacturing technology has focused on bioreactor technology. A bioreactor is simply a controlled environment in which a tissue-like construct can be grown [44]. The design of a bioreactor involves a number of critical issues such as the configuration of the bioreactor, its mass transport characteristics, and its scalability. Hence an important issue in developing a tissue engineered product is how much of the maturation of a substitute is done in vitro in a bioreactor as compared to what is done in vivo through the remodeling that takes place within the body itself.

5.3 Skin

A tissue engineered product replacing skin should principally contain these factors:
- the extracellular matrix;
- dermal fibroblasts;
- the epidermis;
- a semipermeable membrane (between dermis and epidermis).

These components may act synergistically as part of a fully integrated tissue to protect the underlying tissues of a wound bed and to direct healing of the wound. Dermis containing fibroblasts may be necessary for the maintenance of the epidermal cell population. Actually, tissue-engineered skin products on the market would be distinct in cellular-based and acellular products.

The immediate wound coverage is one of the needs of wound management. Engineered epidermal constructs with qualities similar to those of autologous skin have been used to facilitate repair of split-thickness wounds.

Autologous cultured keratinocyte grafts have been used in humans since the 1980s. As a result there has been extensive experience with cultured epidermal grafts for the treatment of burns as well as other acute and chronic wounds [45]. Although they act as permanent wound coverage, since the host does not reject them, disadvantages include the two to three-week time interval required before sufficient quantities of keratinocytes are available.

Cultured keratinocyte allografts were developed to overcome the need for biopsy and cultivation to produce autologous grafts and the long lag period between epidermal harvest and graft production. Cultured epidermal cells from both cadavers and adult donors have been used for the treatment of burns [46]. Although a previous study showed that allografts made from neonatal foreskin keratinocytes were

more metabolically active than those from cadaver, a recent study has shown that such allografts are immunogenic [47]. As an alternative, a chemically modified hyaluronan membrane acting as keratinocyte delivery systems was developed. In this graft cells were delivered to the injury site via a biodegradable scaffold.

Keeping in mind that good skin regeneration requires an appropriate dermal layer, allografts (containing dermis) from other sources have been used for many years, although they provide only temporary coverage due to their tendency to induce acute inflammation. However, this skin can be chemically treated to remove the antigenic epidermal cellular elements and has been used alone or in combination with cultured autologous keratinocytes for closure of various chronic wounds and burns. In spite of these modifications, allogeneic grafts, when compared with autologous grafts, have been shown to promote lower percentages of re-epithelialization and excessive wound contraction [48].

An acellular composite skin graft containing bovine collagen and chondroitin-6-sulfate with an outer silicone covering was developed in the 1980s. After placement on the wound, the acellular dermal component recruits the host dermal fibroblasts while undergoing simultaneous degradation. About two to three weeks later, the silicone sheet is removed and covered with an autograft. This composite graft has been used successfully to treat burns [49]. However, these constructs cannot be used in patients who are allergic to bovine products.

Another type of dermal substitute consists of an inner nylon mesh in which human fibroblasts are embedded, together with an outer silicone layer. After an appropriate time, fibroblasts are lysed in the final product by freeze-thawing. Prior to that time, fibroblasts produce autologous collagen, matrix proteins, and cytokines, all of which promote wound healing by the host.

This product has been used successfully as a temporary wound coverage after excision of burn wounds, until the appearance of the modified product on the market. The new graft contains a biodegradable polyglactin mesh, in which fibroblasts retain viability, instead of the nylon mesh. The use of this dermal substitute has had limited success in the treatment of diabetic foot ulcers, owing largely to its inability to form stable adhesions with the final epidermal graft [50].

Full-thickness wounds involve the loss of both the epidermal and dermal layers of the skin. To treat such extensive wounds, a bilayered skin composite was developed consisting of a collagen sponge containing dermal fibroblasts covered with epidermal cells. A subsequent amendment containing type I bovine collagen and live allogeneic human skin fibroblasts and keratinocytes has been developed. It has been used successfully in surgical wounds and venous ulcers [51]. In a multicenter trial, this product produced accelerated healing of chronic nonhealing venous stasis ulcers when compared to standard compressive therapy [52].

Several other composite skin substitutes combining dermal and epidermal elements have been developed. Composite cultured skin composed of an overlay of stratified neonatal keratinocytes on fibroblasts embedded in distinct layers of bovine type I collagen is currently being evaluated in clinical trials for the treatment of burns.

5

5.4 Lung Epithelium

P. Spitalieri, M.C. Quitadamo, F.C. Sangiuolo

The adult lung is lined with numerous distinct types of epithelial cells in various anatomical regions, progressing proximally in the order trachea → bronchi → bronchioles → alveoli. The conducting airways can be further divided into two major compartments, the tracheo-bronchial and bronchiolar airways, while the alveolar regions of the lung make up the gas exchanging airspaces. Each of these regions consists of distinct epithelial cell types with unique cellular physiologies and stem cell compartments. In a normal steady state airway, epithelial cell proliferation in the adult lung is much lower compared with highly proliferative compartments in the skin, gut, and hematopoietic system. As in other adult tissues and organs, stem cells in the adult lung are a subset of undifferentiated cells with the capacity to maintain multipotency in the context of the physiologic domain in which they reside. Experimental studies [53] in animals have shown the presence of different types of stem cells along the airways, but regardless of the dominant cell type, endogenous repair is not sufficient for the prevention of respiratory diseases and is thus a major cause of human mortality and morbidity worldwide. Current treatments offer no prospect of cure or disease reversal, and lung transplantation is an acceptable means of treating several end-stage lung disease, even though donor shortage is a major problem. Over the past few years, the number of lung transplantations performed annually has reached a plateau as the donor pool is approaching its limits and cannot be expanded further. Therefore other sources of a replacement gas-exchange units are being sought. Xenotransplantation [54] and bioartificial devices [55] are possible candidates. Another possibility would be to engineer a gas-exchange unit with some viable components in vitro. Alveoli are the basic functional units of lungs, and gas exchange occurs across their membranes. Therefore, the first step in tissue engineering of a lung lobe needs to be aimed at the generation of alveolar tissue. The epithelium of an alveolus is an endoderm-derived tissue composed of two main cell types: type I (ATICs) and type II (ATIICs) pneumocytes. The ATICs are large yet highly flattened cells with multiple apical surfaces that extend into adjacent alveoli. These cells, together with the endothelium of the surrounding capillaries, form the very thin blood–air interface that is essential for O_2/CO_2 exchange. In contrast, ATIICs are small cuboidal cells that secrete surfactant, which reduces surface tension, preventing collapse of the alveolus. It is known that the lung is constantly exposed to environmental toxins and pathogens that can destroy alveolar epithelial cells, in particular the thin injury-prone ATICs. If ATI cells are lost after a peripheral lung injury, ATII cells undergo proliferation and differentiation to the type I phenotype. Thus, ATII cells are crucial to the natural regenerative process of the alveoli. In fact, ATII cells are considered as putative alveolar stem cells [56]. Therefore, generation of ATII pneumocytes would be a good basis for tissue engineering of the alveolar functional unit [57].

Inadequate, delayed, or impaired re-epithelialization of the injured alveolus is

regarded as a key factor in the pathogenesis of several life-threatening pulmonary diseases, including acute lung injury, acute respiratory distress syndrome, and chronic obstructive pulmonary disease. Severe pulmonary diseases can be caused by deficiencies or genetic mutations in proteins synthesized by ATII cells that are important in maintaining normal lung homeostasis. For example, complete deficiency of surfactant protein B (SPB) is caused by genetic mutations in the SPB gene. This deficiency results in impaired pulmonary surfactant composition and function and is a major cause of fatal neonatal respiratory disease. Cystic fibrosis is thought to be primarily a disease of the upper airway and submucosal epithelia and is caused by mutations in the cystic fibrosis transmembrane conductance receptor (CFTR) [58]. CFTR is an important regulator of Cl- and liquid transport in the lung and is functionally expressed by human ATII cells, strongly suggesting a critical role for CFTR in regulating ion and fluid transport in the lung alveolus in addition to the upper airway. Current treatments for lung alveolar epithelial injury at best provide symptomatic relief but offer no prospect for repair of the damaged epithelium or preventing lung fibrosis. Consequently, there is a pressing need for the development of novel therapies that facilitate the regeneration of alveolar epithelium destroyed by acute and chronic lung diseases.

Embryonic stem cells (ESCs) are self-renewing pluripotent cells, which can be induced to differentiate into a wide range of different cell types. Recently published data has demonstrated that ESCs cells can be differentiated into ATII cells via embryonic body (EB) formation [57] or coculture of EBs with pulmonary mesenchyme [59]. ATIICs derived from ESCs may be promising as a transplantable source of cells that could be used therapeutically to treat distal lung injury [60]. In fact lung progenitor ATIICs derived from human ESCs can be transplanted into a mice model of bleomycin-induced acutely damaged alveoli and that these hES-ATIICs arrested or reversed BLM-induced pathological changes of acute lung injury, including fibrosis. Moreover, the therapeutic benefits provided by the transplantation of hES-ATIICs were long- term and without the development of teratomas [61].

Recently, a new source of human stem cells was isolated from amniotic fluid (hAFSC) [62] and umbilical cord blood (uMSCs) [63]. These are multipotent, showing the ability to be integrated into developing as well as injured lung tissues and differentiate therein into lung epithelial lineages. Other authors have shown the in vitro ability of the fetal human umbilical cord collected from pre-term newborns (HUCPC) to migrate towards an alveolar type II cell line damaged with bleomycin, an anticancer agent with known pulmonary toxicity. The secretory profile exhibited by fetal HUCPC in the migration assay suggested a paracrine effect that could be exploited in various clinical conditions including lung disorders [64]. These sources of stem cells could overcome all of the ethical issues concerning use of embryonic stem cells. The possibility of deriving specific lung lineages from stem cells in vitro has already been shown, as well as evidence that allogeneic and xenogeneic transplantation of lung epithelium cells can reduce bleomycin-induced lung fibrosis in mice after intratracheal and/or intraperitoneal injection [65]. Additional experiments and different approaches are needed to optimize hES-ATIICs integration, survival and recovery of damage in mouse-model in vivo to then move on to clinical

trials on humans for cell-based therapy, and further studies toward the use of these cells for the repair of tissue damage associated with inflammatory and fibrotic degeneration are warranted.

5.5 The Bioartificial Liver

G. Resta, C. Rossi, G. Azzena

The bioartificial liver (BAL) is an extracorporeal circulation therapeutic system consisting of two components, an artificial one (bioreactor) and a biological one (hepatocytes). This system was created for the temporary treatment of patients with acute liver failure during the wait for spontaneous recovery or an organ transplant. Despite the increased production of the number of BAL and their current experimentation, few models have been used for clinical applications.

5.5.1 Introduction

Acute liver failure (ALF) is a clinical heterogeneous entity in terms of etiology, pathophysiology, severity and prognosis. ALF can be divided into fulminant hepatic failure (FHF), which represents the most severe form; acute liver failure occurring in chronic hepatitis (acute on chronic liver failure, AoCLF) and liver failure after liver transplantation (Primary non function, PNF). Despite considerable progress in understanding the pathophysiologic mechanisms leading hepatic failure, the birth of special liver intensive care units and the implementation of various therapies, the mortality rate for FHF today still varies from 50% to 80% cause depending. In Italy it is estimated that there are about 300-350 FHF cases each year, while the figure in the United States is about 2000-3000 cases each year. Although liver transplantation has radically revolutionized the therapy of this disease, unfortunately this method is limited by the number of donated organs. For this reason there is a need to experiment alternative approaches for liver support, which can assist the patient with ALF until the spontaneous recovery of function or the retrieval of an organ for transplantation. Traditional medical and pharmacologic support were therefore flanked by artificial biological and bioartificial liver support techniques. The artificial support techniques consist of cell-free systems that exploit detoxifying principles of adsorption and filtration to remove circulating toxic substances. One interesting application is MARS, which exploits the ability of albumin to bind the toxic substances present in the plasma of a patient with severe renal failure. Although this method has shown an increase in patients survival with AoCLF, it has not demonstrated improved survival in patients with fulminant hepatitis. According to many authors, the main limits of artificial systems are due to their inability to substitute the metabolic and synthetic liver component.

Recently the most studied technique of biological support has been the transplantation of hepatocytes. Though promising, this method is still preclinical, since in literature there are only few clinical experiences, most of which being case reports.

5.5.2 Bioartificial Technical Support

BAL is composed of a biological component and an artificial one. These two components together exploit the detoxifying skills of the artificial systems and the synthetic-metabolic ability of the liver cell. The biological component may be derived from an isolation cell enzyme or cell lines. The cells are then incubated on three-dimensional supports inside bioreactors of different models, but all these bioreactors have as a general principle the creation of two separate compartments: the cellular compartment for hepatocytes and the circulation compartment for plasma or human blood. The two compartments are separated by physical means which act as semipermeable barriers, which allow the passage of toxic substances and chemical components derived from cellular metabolism.

5.5.3 BAL Structure

The various bioreactors used differ in terms of cell types, circuitry and materials used. Our group has developed its experience using the Performer O. Liver BAL, produced in Italy in cooperation with the firm Rand Srl of Medolla (MO). The artificial part of the O. Liver BAL consists of a plasmapheresis machine (Performer BAL), an extracorporeal circuit for the perfusion of hepatocytes, a bioreactor inside of which the cells are contained, a plasma oxygenator for the perfusion of the hepatocytes and a resin cartridge which selectively adsorbs bilirubin and bile acids (Fig. 5.1). The extracorporeal circuit is connected to the patient through a double lumen catheter inserted into the superficial femoral vein. The Performer BAL is a complex device consisting of pumps for extracorporeal circulation, connected to filters in hollow fibers of polypropylene (0.2 m^2 of total strained surface) for the plasma separation with a cut-off of 0.4 mm. Using a plasma reservoir with a capacity of 2 L and an additional peristaltic pump, a second circuit allows the plasma circulation through the oxygenator, the bioreactor and the cartridge of synthetic resin, with a speed of 200-250 mL/min. Oxygenation of the hepatocytes is maintained through an oxygenator integrated in the circuit which under conditions of clinical use supplies a mixture composed by O_2 (95%) and CO_2 (5%). The flow of blood is usually maintained at 80±20 mL/min, while the flow of plasma is 22±8 mL/min. In the liver support system in question, we used an adsorbent cartridge containing a copolymer (artificial resin) with porosity of 300-1000 μm, with the aim of replacing the lack of an excretory bile tract in BAL. For our model of BAL a bioreactor (O. Liver

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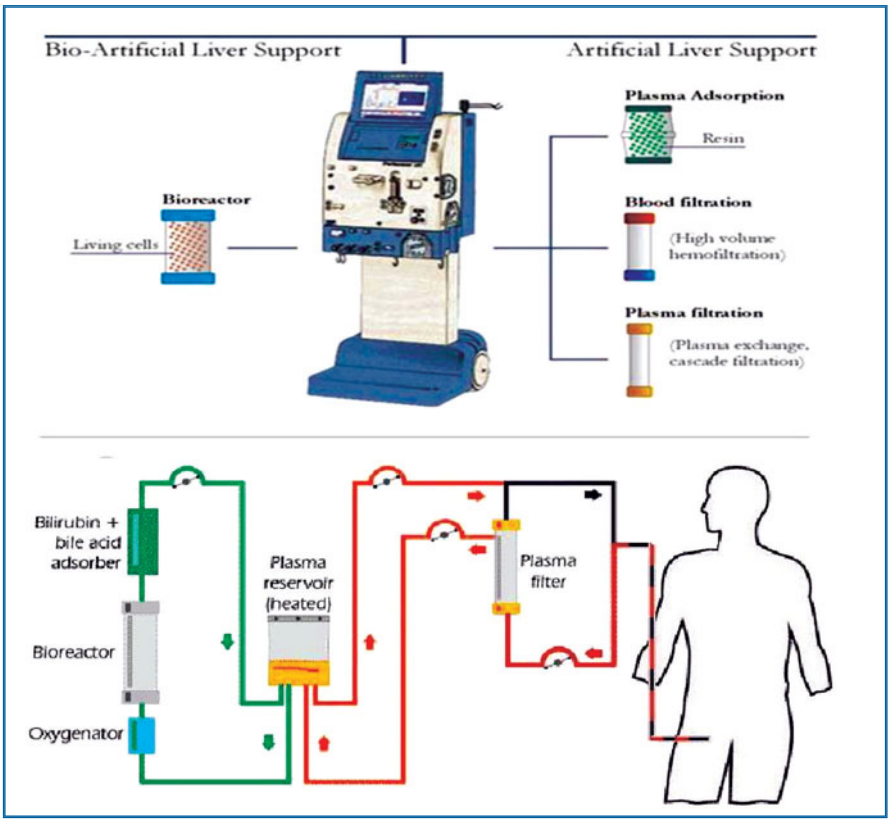

Fig. 5.1 Performer BAL and extracorporeal circuit. (Courtesy of RanD S.r.l.)

cart-A, RanD S.r.l., Cavezzo, MO, Italy) has been developed specifically designed for use with liver cells. The principle on which it has been designed is to allow direct contact between the patient's plasma and liver cells. For this reason the geometry of radial flow was adopted, according to which the plasma diffuses from inside to outside the bioreactor, after passing through the compartment occupied by the cells. The porosity of the polyester membrane (1 micron) allows a broad exchange of solutes and molecules but not the escape into the circulation of liver cells contained in the bioreactor, neither during the filling phase of the bioreactor with hepatocytes, nor during subsequent clinical use.

5.5.4 Clinical-Laboratory Parameters Assessed

The evaluation of patients with fulminate hepatitis and treated with BAL provides an analysis of clinical parameters (such as neurologic function) and blood chemistry

parameters (blood count, liver and kidney profile, blood clotting and blood ammonium). At the same time we carry out an investigation on the functionality of the bioreactor, especially with the execution of sequential blood gas analysis on plasma entering and leaving the bioreactor.

5.5.5 Clinical Trials

Many clinical applications have been conducted to date, some with excellent results [66-68]. At the Institute Anphioxus Cell Technologies of Houston 11 patients were treated using hepatocyte cell lines (C3A). In these 11 patients there was an average improvement of bio-humoral parameters and in 10 of them an improvement of neurological presentation. In Pittsburgh 4 patients with fulminant hepatitis were treated, using porcine hepatocytes, with a decrease in ammoniemia during treatment being reported, but no improvement from a neurological point of view. Even in China, at the Chinese PLA General Hospital of Beijing clinical applications have been conducted of BAL loaded with porcine hepatocytes in 3 patients with fulminant hepatitis, with a decrease n ammoniemia and an improvement of neurological presentation during treatment being reported. In Germany, at the Charité, Humboldt University of Berlin 7 patients with fulminant hepatitis were treated using a BAL loaded with porcine hepatocytes. All the patients received a transplant operation, while not showing a sharp decrease in ammoniemia during treatment. At the same center, 4 patients with fulminant hepatitis were recently treated using BAL loaded with human hepatocytes from organs rejected by transplant. All patients showed an improvement of neurological conditions, but a worsening of coagulation during treatment. In Rome and Naples BAL loaded with porcine hepatocytes assembled at the Academic Medical Center University of Amsterdam was used to treat 12 patients with FHF. Improvements in neurological conditions during treatment were also described in this case. Demetriou et al. in Los Angeles conducted the largest prospective, randomized, controlled, multicenter trial in patients with severe hepatic failure treated with the BAL loaded with porcine hepatocytes (Hepatassist System) [69]. The study was conducted on 171 patients of whom 85 treated with BAL and 86 patients as a control. This trial showed the safety and effectiveness in terms of survival of BAL treatment. At our Institute of Ferrara 9 patients with fulminant hepatitis were treated using a BAL (O. Liver) loaded with fresh porcine hepatocytes in 7 patients [70] or human hepatocytes from organs deemed unsuitable for transplant in 2 patients. Of these, 7 patients received transplant after treatment with only one death. One died while waiting for a transplant and another showed a spontaneous recovery of liver functions. In our case we also evaluated improvement of the neurological situation as well as the bio-humoral parameters.

5.5.6 Conclusions

Although BAL has been shown to have a therapeutic effect, the case reports published up to now are not enough to say with certainty that this method may produce a significant increase in survival in patients with severe liver failure waiting for transplant. In addition, various bioartificial livers adopted to date by different researchers differ in terms of the extracorporeal systems used and the cell source. It is logical to think that the fresh cells, derived from healthy and young organs would be the ideal source for this type of clinical application. It is possible to obtain the above using porcine cells isolated and used fresh, as we did in the first 7 clinical cases described. In many European countries, however, like in Italy, the use of animal cells is banned for health safety reasons. Therefore alternative sources need to be used, such as human cells derived from organs deemed unsuitable for transplantation. However, the inability to match the emergency clinic with the presence of an immediately available organ prompts the need to establish a bank capable of cryopreserving these cells. On the other hand, although human cryopreserved cells have shown in vitro a specific detoxifying activity and different biosynthetic activities, when used within the bioreactor they have demonstrated limits in the biosynthesis and duration of functional hepatocyte activity. The causes of this limitation obviously includes the cell microenvironment, consisting of the toxic patient's plasma, which does not facilitate the functional recovery of hepatocytes already stressed by the processes of isolation, cryopreservation, thawing and loading in the BAL. A significant evolution could be represented by the joint and integrated use of artificial and bioartificial systems, with the first component having the role of selective detoxification and the second component the role of synthesis-metabolism. Moreover, if we continue to use human cells, it is essential to increase knowledge about the cryopreservation phenomenon, to have available a cell pool as vital and functional as possible and ensure the best performances when used within the BAL. Many research groups are increasingly tending towards more complex bioengineering, with a reduction in the artificial component in favor of the biological one. This will develop real liver organelles, the function of which is still disputed. Even at our center we have performed studies on organelles consisting of porcine hepatocytes encapsulated in a matrix of calcium alginate (Fig. 5.2). Such studies now have a role of preclinical testing in vitro, although with encouraging preliminary results.

In conclusion, it should be borne in mind that ALF currently causes more than 30,000 deaths in Europe each year, ranking it the eighth cause of death. So there is a constant need to work harder to develop valid liver support systems as an alternative to or interim procedure in the wait for liver transplantation. A more wide consensus will be necessary in future clinical trials on BAL, particularly in the choice of objectives, controls and indications to treatment. It is therefore desirable to implement controlled trials in phase II and III to arrive later to clinical application over a larger number of patients who still today die while waiting for transplant.

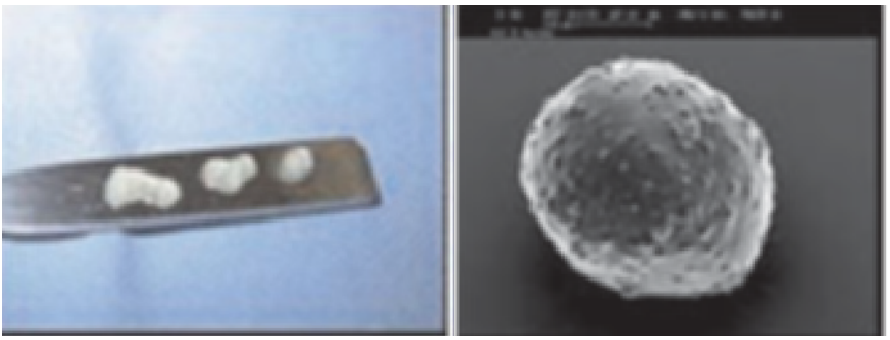

Fig. 5.2 Hepatocyte organelles in calcium alginate (Center for Development of Advanced Cell Therapy, University of Ferrara)

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In Vivo Imaging of Regenerated Tissue: State of Art and Future Perspectives

6

V. Lionetti, A. Pingitore

6.1 Introduction

Mesenchymal stem cells (MSCs) are multipotent cells which can give rise to mesenchymal and nonmesenchymal tissues in vitro and in vivo [1]. The distribution of resident MSCs throughout the post-natal organism is mainly related to their existence in perivascular niches [2]. They can differentiate into osteogenic, adipogenic, chondrogenic, myocardial, or neural lineages when exposed to specific stimuli, making them attractive for tissue regeneration [3, 4]. Emerging evidence has shown that MSC transplantation offers a means to stimulate tissue repair either by direct (exogenous) or indirect (endogenous) cell replacement or angiogenesis [5, 6]. In fact, exogenous MSCs have shown the ability to support a paracrine activation of endogenous stem cells for tissue repair by secreting chemokines, as stromal derived factor-1 alpha (SDF-1α), and/or growth factors, as vascular endothelial growth factor. Despite the rapid research advancement, possible tissue repair by adult stem cell therapy is currently hampered in vivo by poor cell viability and delivery efficiency, uncertain differentiating fate, and therefore the use of this approach has raised a number of bioethical questions [7]. Hence, the strong need for more effective therapeutic approaches emphasizing the physiological plasticity of postnatal organs following an injury [8, 9], and more accurate imaging methods to allow a long-term in vivo monitoring of tissue regeneration [10]. Indeed, one of the most important accomplishments of modern physiology is the development of imaging techniques able to explore biochemical/molecular processes in the intact organism, i.e. in the absence of confounding effects inevitably caused by invasive procedures or ex vivo experimental preparations. Modern imaging technologies allow for

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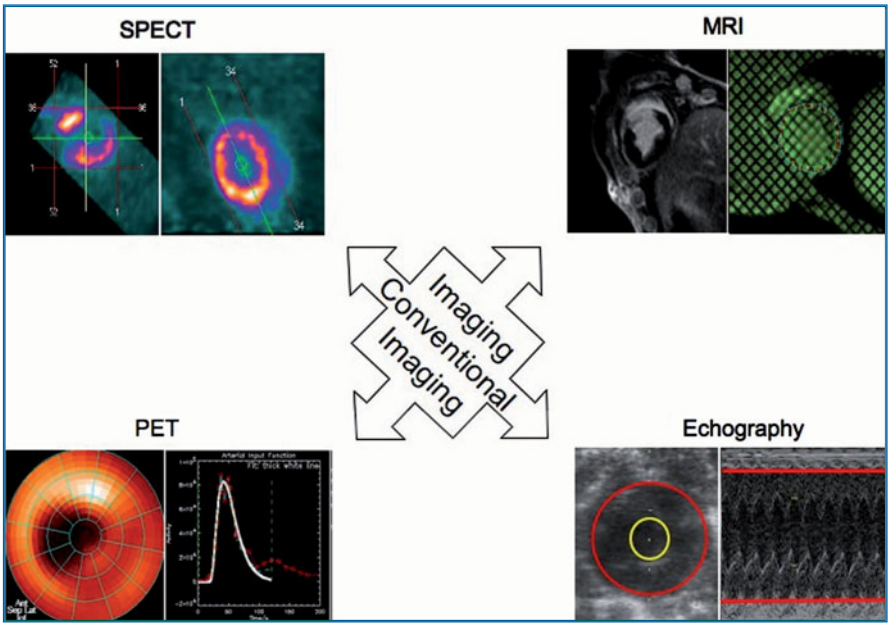

Fig. 6.1 Conventional noninvasive imaging techniques in cardiac regeneration

visualization of multidimensional and multiparameter data. Imaging is increasingly used to measure physical parameters, such as concentration, tissue properties, and surface area, and to glean temporal insight on biological functions. With the advent of molecular probes, imaging can be conducted not only to visualize gross anatomical structures, but also to visualize substructures of cells and monitor molecule dynamics. Thus, different modalities can be used for non-invasive conventional and molecular imaging of regenerated tissues in vivo, such as positron emission tomography (PET), single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI). Moreover, targeted and conventional ultrasound modality is even starting to play a role in regenerative medicine due to resolution, low-cost and availability compared to SPECT, PET, MRI, computed tomography (CT) and optical imaging [11]. The translation of new integrated imaging approaches into effective noninvasive follow-up of tissue repair is emerging as an outstanding area of progress for regenerative medicine over the next decades and has already found broad application in cardiac cell therapy (Fig. 6.1).

6.2 Conventional Imaging of Regenerated Tissue

The regenerative process involves the replacement of dysfunctional and/or fibrotic tissue with differentiated and organ-specific new cells, well organized both from a

structural that functional aspect, also in response to extracellular matrix (ECM) degradation products [12]. The regenerated tissue needs to be perfused by a network of well differentiated neovessels branching within the ECM and functionally adapted to the resident vascular architecture [13]. PET modality is the gold standard for noninvasive assessment of tissue viability and allows an accurate detection of vascular network by measurement of tissue blood flow, respectively through the intravascular injection of 18F-fluorodeoxyglucose (18F-FDG) or C-11-acetate [14] and 13N-labeled ammonia ($13NH_3$) [15]. Other PET radiotracers are currently under investigation to visualize cell proliferation within viable tissue, such as 3'-deoxy-3'-[18F]-fluorothymidine [16] which could be very useful in the potential monitoring of the activated resident stem cells during regeneration. Recent studies demonstrated the utility of PET in visualizing the tissue before and after a regenerative therapy in different types of tissue, such as the heart [17] and the nervous system [18]. Although radiotracers visualize certain normal anatomical structures, the spatial resolution is generally inadequate for accurate anatomic localization of the treated region. Conversely, MRI provides high resolution anatomical images that allow accurate regional evaluation of tissue structure and function, detection of interstitial fibrosis following cell therapy in the heart [19], cartilage [20] and nervous tissue [21]. MRI is a highly versatile imaging technique that enables different applications to be performed depending on the type of study. Functional MRI (fMRI), a specialized MRI application, is able to measure the hemodynamic response (changes in blood flow) related to neural activity in the brain or spinal cord. It might provide new insights into the role of adaptive changes in limiting the clinical consequences of irreversible structural damage. In fact, when nerve cells are active they increase their consumption of oxygen, switching to more rapid anaerobic glycolysis, and thus the regional blood flow increases [22]. Furthermore, MRI could also be a sensitive and efficient tool to study tissue metabolism in vivo using hyperpolarized 13-C pyruvate, a well known substrate of oxidative metabolism [23]. As a well established index of cell viability in vivo, pyruvate metabolism might therefore particularly benefit from noninvasive regional imaging of regenerated and injured tissue. Combining PET with a high-resolution anatomic imaging modality, such as CT or MRI, can resolve the localization issue as long as the images from the two modalities are accurately coregistered [24]. Compared to two stand-alone machines, the combination of a PET and CT/MRI scanner into a single gantry also provides greater convenience, flexibility, and speed for multimodality imaging of regenerated tissue, which would seem like normal tissue. In this regard, a recent study by Lionetti et al. [8] showed that the regenerated myocardium in a rodent model of myocardial infarction is characterized by normalization of 18F-FDG myocardial uptake, assessed by mini-PET, reduction of fibrosis, detected by gadolinium delayed-enhancement MRI, and improvement of left ventricular remodeling and contractile function.

6.3 Molecular Imaging of Regenerated Tissue

Molecular imaging describes a heterogeneous family of noninvasive imaging techniques that have been developed for use in both basic and clinical settings. This modality encompasses the myriad of different optical techniques that have been developed to profile cell properties, such as protein expression, cellular and molecular trafficking, gene expression, enzyme activity, and pH. The techniques employed for these diverse applications differ in terms of the capabilities of the detectors (spatial resolution and sensitivity); properties of the imaging probes (toxicity, bio-distribution and specificity for target); and practical issues such as image acquisition time and costs [25]. Ultimately, the use of a molecularly targeted nanoplatform affords many advantages over established approaches. First, more imaging labels or a combination of labels for different imaging modalities can be attached to a single nanoparticle, which can lead to merge different signals from the same target tissue in vivo. Second, multiple, potentially different, targeting ligands on the nanoparticle can provide enhanced binding affinity and specificity. Third, the ability to integrate a means to bypass biological barriers can enable enhanced targeting efficacy [26].

The visualization of biological processes in living systems, such as regeneration and repair of injured tissue, has the main expectation in localization and quantification of molecular changes associated with a disease at an early stage rather than later when morphological changes have occurred [27]. For this purpose, the application of nanotechnology to imaging allows transplanted stem cells to be tracked and guided, as well as understanding the roles of paracrine signals following the secretion of soluble factors, the cell-cell interactions or the action of physical forces. An innovative approach to harnessing the respective strengths of various imaging platforms is the creation and use of specific contrast agents [26] and reporter genes [28]. Fusion reporter constructs, such as red fluorescent protein (RFP) [29], firefly luciferase (fluc) [28], herpes simplex virus thymidine kinase (HSV-tk) [30] and heavy chain of ferritin (FTH) [31], have been identified as potential tools in molecular imaging of regenerated tissue (Fig. 6.2). Non-invasive molecular imaging of dynamic processes, such as tissue regeneration, has benefited tremendously from the use of reporter genes encoding for proteins that emit light, bind radiolabeled probes, or modulate MRI contrast. Indeed, the above constructs mainly make available the use of the following imaging modalities: optical fluorescence, bioluminescence, PET and MRI. In addition, contrast-enhanced ultrasound (CEU) techniques using site-specific microbubbles have been developed for the molecular imaging of the vascular phenotype, which characterizes neoangiogenesis, and to visualize progenitor cell engraftment into new vessels and interstitial spaces [32].

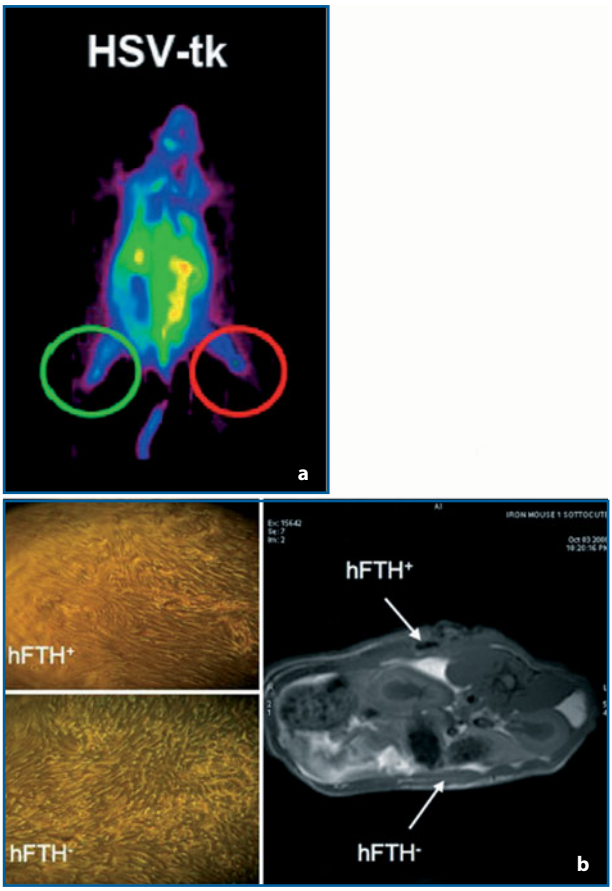

Fig. 6 2 Reporter genes in vivo. **a** PET image of stem cells labeled with herpes simplex virus thymidine kinase (HSV-tk) injected in skeletal muscle (*green circle*) compared to untreated leg (*orange circle*); **b** MRI T2* image of stem cells labeled with human heavy chain ferritin (hFTH+) injected subcutaneously in rats

6.3.1 MRI Molecular Imaging: Contrast Agents and Reporter Genes

In providing information regarding the transplanted stem cells, MRI offers several advantages, such as high resolution, speed, easy accessibility and 3D capabilities. A significant advantage of MRI is that it provides information regarding the surrounding tissues (e.g. edema, lesion or inflammation), which may have an effect on the fate of grafted stem cells or may hinder the recovery of damaged tissues. Due to its biocompatibility and strong effects on T2(*) relaxation, iron oxide nanoparticles (SPIONs) appear to be the contrast agent of choice, and several methods now exist to shuttle sufficient amounts of these compounds into stem cells. Dextran and other polymer-coated SPIONs are currently used in a number of biomedical applications [33]. Stem/progenitor cells can be acutely labeled with dextran-coated SPIONs by modifying their surfaces with internalizing ligands, such as the HIV-Tat peptide,

dendrimers and polycationic transfection agents [34]. In particular, dendrimer composites of iron oxide nanoparticles, also known as magnetodendrimers, represent a versatile new class of contrast agent for MRI. They were developed by Bulte et al. and label living cells efficiently, including stem/progenitor cells without affecting their phenotype and growth rate [35]. In a recent study, Wang et al. labeled neural stem cells with gold-coated monocrystalline SPIONs [36]. The MRI signals persisted 1 month postsurgery and the gold surface protected the nanoparticles from being digested by the macrophages. It was concluded that gold-coated SPIONs may represent a class of superior MRI labels for long-term in vivo tracking of stem cells. Despite these promising results, the contrast agents listed above do not allow a stable visualization of stem cell-derived cells or dividing cells in vivo. For this purpose, it is conceivable to label stem cells with reporter genes [37].

Several strategies exist to modulate MRI contrast by expression of reporter gene into transplanted stem cells: using enzyme-catalyzed chemical modification of metal-based contrast agents or (phosphorus) metabolites, such as creatine kinase, an enzyme that catalyzes ATP conversion to ADP producing phosphocreatine (PCr), detected by 31P magnetic resonance spectroscopy [38]; iron-binding and iron-storage proteins to accumulate iron as a contrast agent, such as transferrin receptor [39] and heavy chain of ferritin [40]; artificial proteins for imaging based on chemical exchange saturation transfer (CEST), such as a lysine rich protein (LRP), containing a high density of amide protons [37]. Ultimately, CEST contrast agents have two major advantages. First, they are switchable, i.e. the contrast is detectable only when a saturation pulse is applied at the specific frequency characteristic of an agent's exchangeable protons. Otherwise, the contrast agent is MRI-invisible. The second advantage is that different contrast agents with different excitation frequencies can be used for imaging simultaneously more than one target. This property might be exploited for imaging multiple cells (e.g. for studying neuronal circuits) or the expression of multiple genes (such as in genetic circuits). Despite the technological development, MRI reporter genes need further improvement to facilitate their wider use to track stem cells and follow their specific differentiation during regeneration of injured tissue in vivo. In addition, the acquisition of more specific reporter-induced MRI contrast may be achieved using optimized pulse sequences and improved ways of image processing. Reporter genes might be improved through directed evolution in a fashion similar to that accomplished for the fluorescent reporter genes. A key challenge would be to resolve multiple targets while performing simultaneous imaging. This could include different reporters driven by different inducible promoters or developing sophisticated data processing algorithms to distinguish gene expression temporally as well as spatially. However, from the clinical point of view there are still a few significant hurdles before reporter genes can be introduced into the human body.

6.4 Conclusions

With the rapid increase of reported cases of stem cells being used to treat tissue injury, it has become apparent that an urgent need exists to track stem cells in vivo during clinical trials. The problem of imaging small numbers of cells in the living subject is not limited to stem-cell-based treatments in cardiology, but has broad applicability in oncology, surgery, and transplantation. Successful in vivo imaging requires that a contrast agent or reporter genes associated with a stem cell exert an *effect size* sufficient for detection by imaging hardware. In addition, the exogenous contrast agent and reporter genes must be biocompatible, safe, and nontoxic. Imaging technology has also a significant clinical relevance, but no currently available imaging technology is ideal. MRI provides excellent 3-dimensional anatomy but some contrast techniques have low sensitivity. However, multimodality contrast agents might improve the prospects for stem cell tracking both in vivo and ex vivo in regenerative medicine. First and foremost, impending clinical trials utilizing stem cells must carefully define the limits of the imaging technology chosen.

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Biotechnological Approaches to Hemostasis and Molecular Mechanisms of Wound Healing

7

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7.1 Biotechnology for Hemostasis Control

Wounds today remain a difficult clinical problem due to early or late complications that are a frequent cause of mortality. To try to resolve this problem, many studies have focused on understanding physiologic healing and wound care, with particular emphasis on new therapeutic approaches and the continued development of biotechnology for the management of acute and long term injuries. Normal wound healing is a dynamic and complex process involving a series of coordinated events, including bleeding, coagulation, initiation of an acute inflammatory response to the initial injury, regeneration, migration and proliferation of connective tissue and parenchyma cells, as well as synthesis of extracellular matrix proteins, remodeling of new parenchyma and connective tissue and collagen deposition. Lastly, increasing wound strength takes place in an ordered manner and culminates in the repair of severed tissues [1].

Hemostasis is the first event involved in wound healing; it is an ensemble of biotechnology processes which act to prevent or stop both arterial and venous bleeding, with subsequent lesion repair in blood vessels and restoration of the functionality of damaged tissue. Hemostasis occurs spontaneously through the coagulation processes, or can be mechanically induced in surgery, which is the crucial part of surgery.

The process of coagulation and subsequent tissue repair can be broken down into four basic steps:

- when vascular trauma occurs, there is a reflex constriction to restrict blood flow;

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- subsequently the platelets are activated by thrombin. They aggregate at the site of the lesion and form a temporary plug. The protein fibrinogen is primarily responsible for platelet aggregation. When platelets are activated, several important factors in the coagulation cascade are released, such as the nucleotide ADP, the eicosanoid TXA2, serotonin, phospholipids and lipoproteins. Moreover, platelets also change their shape to accommodate plug formation;
- at this point a fibrin network is formed, known as a clot, which traps the plug to ensure stability;
- lastly the clot must be dissolved to allow normal blood flow once tissue repair has occurred. This is done by plasmin [2].

There are two pathways for fibrin clot formation: the intrinsic and extrinsic pathway. The intrinsic pathway is mainly activated by exposed collagen, following vessel rupture. It binds to factor XII and the coagulation cascade starts. The extrinsic pathway instead is activated by tissue factor, which is released after tissue injury; it forms an activated complex with factor VII to initiate coagulation processes. Both pathways converge in a common pathway, where thrombin converts fibrinogen into fibrin monomers, which cross-link with the help of factor XIII and calcium to form a fibrin polymer and then the clot [2].

7.2 Hemostatic Agents and their Mechanism of Action

Adequate hemostasis is a prerequisite for the success of any surgical procedure [2]. Hemorrhages and bleeding that occur during surgery are usually due to ineffective local hemostasis, although hemostatic defects arising from the patient can occur. Currently, the procedures used in surgery for hemostatic control can be chemical, mechanical and thermal. However, these systems may not be sufficient for good hemostasis, so much so that the search for biomaterials that can repair damaged tissues and simultaneously stop the bleeding is intense. Of course, these agents should not be considered substitutes for meticulous surgical practices.

Biomaterials may have a biological nature such as blood products, a natural source such as collagen, or they may be of synthetic origin such as polymers of various substances, chemical composition and physical appearance. They must have basic characteristics to be useful in surgery, such as bio-reabsorption, biodegradation, biofunctionality, sterility and lack of immunogenic reaction from foreign bodies [3].

Biomaterials that support surgery in hemostasis resolution can be divided into topical hemostats, sealants, and adhesives. Hemostats are capable of clotting blood; sealants can provide a sealing barrier in the presence or absence of blood; and adhesives bond tissues together [4]. Hemostatic agents include collagen, gelatin and cellulose, while an example of a sealant is fibrin glue. Sealants or adhesive can also be produced synthetically (Fig. 7.1).

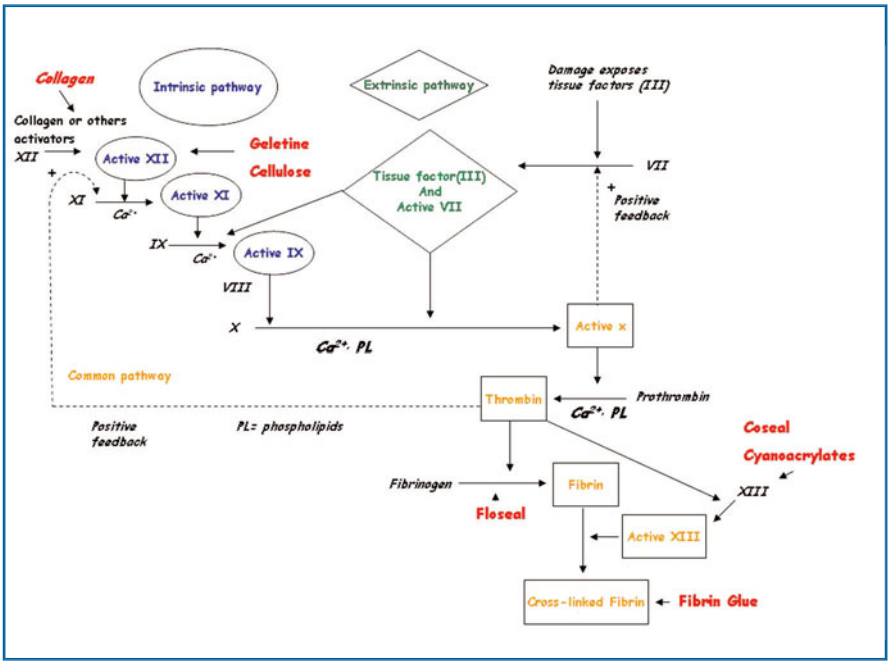

Fig. 7.1 Hemostatic agent: site of action in coagulation cascade

7.2.1 Collagen and Gelatin Sponges

Collagen is derived from bovine collagen and can be produced in fibrillar and microfibrillar forms. These agents act as a matrix for clot formation and strengthening and also reinforce platelet aggregation, degranulation, release of coagulation factors, which combined with the plasma factors produce a fibrin clot. The major concerns with bovine collagen, in term of safety, are swelling with tissue compression, a potential nidus of infection, or adhesion formation. For these reasons, surgeons tend to make limited use of it and remove any excess material. As all bovine collagen products contain small amounts of bovine serum proteins, which may increase immunologic titers, these bovine collagen products should not be used in patients who are allergic to the material. In fact all of these agents can cause foreign body reactions. Their absorption occurs in about 8 to 10 weeks and they should not be used for skin closure as this may prevent healing in the presence of methyl methacrylate glues as they reduce glue adherence, nor should they be used with blood salvage systems because their strands can be smaller than the 40-mm filter size available in such systems. Lastly, these products have a potential for alveolar osteitis in dental extractions and in otolaryngologic surgery. With respect to efficacy, when conventional techniques are ineffective these products may provide hemo-

stasis within 1 to 5 minutes. They are supplied in a variety of sponge sizes and some of the flours are provided in syringes and laparoscopic applicators to enhance the applicability of the product. These products are not to be re-sterilized. They are inactivated by autoclaving and their combination with ethylene oxide produces ethylene chlorohydrin. Both in vitro and in vivo studies suggest that bovine collagen products may be the most efficacious of the mechanical hemostats [5].

The most commonly used hemostatic collagens include Floseal®, Tissufleece® and Gentafleece®. Tissufleece® is a collagen sponge with hemostatic effect but without adhesive properties; with the addition of gentamicin it becomes Gentafleece® [3], a flap of *native* collagen of equine origin that promotes adhesion and aggregation of platelets; it can be stored at room temperature and can also be used in combination with the application of fibrin glue.

Floseal® consists of thrombin and a gelatin matrix produced by the extraction of collagen from bovine corneal tissue. Both components act synergistically to allow clot formation at the bleeding site. Floseal® matrix, in the form of a high viscosity gel, conforms to any type of injury. It can act on all types of bleeding: capillary, venous and arterial. It works in minutes and its absorption typically occurs 6-8 weeks after application. Floseal® also allows the excess product to be removed, and in this way its effectiveness can be verified to ensure a lower risk of foreign body reaction and always ensure the *clean field* at the site of bleeding. The effectiveness of Floseal® has been demonstrated in studies of general, vascular and cardiothoracic surgery [2].

Gelatin sponges are a protein mixture obtained from collagen and materials rich in collagen. They have the ability to dissolve in hot water to form colloidal solutions that, if sufficiently concentrated, cold coagulate into a colloidal solid mass (hydrogel). In the strictly medical field they are used for their hemostatic properties in internal bleeding, by parenteral administration or more often for local application. Thanks to their composition they have the advantage of being completely absorbed by the tissues and can therefore be left in situ during surgery. Typically, the gelatin sponge is used to stop bleeding in the cavity communicating with the outside, because once soaked in blood, despite the addition of antibiotics or antiseptics, it becomes the site of microbial growth.

Spongostan®/Surgifoam® is a gelatine based absorbable hemostat. It absorbs 45-50 times its own weight in blood and within 4-6 weeks it is completely absorbed by the human body. The gelatine sponge is available in different sizes to address specific surgical needs and it can be cut into the desired size or shape without fragmentation. The sponge can be applied dry or moistened with sterile saline or thrombin. Spongostan®/Surgifoam® has an impressive safety record. It has been used in over 100 million surgical procedures throughout the world. The easy preparation, the versatility and the unique safety profile makes Spongostan®/Surgifoam® an excellent choice when a professional and reliable hemostatic product is needed. Spongostan®/Surgifoam® is also available as powder. The powder is mixed with saline or thrombin and after mixing it becomes a pliable mass that can be applied on rough and uneven cut surfaces, it covers them completely and induces hemostasis rapidly. Spongostan® Powder/Surgifoam® Powder is supplied in a ready to use ster-

ile mixing container that makes mixing easier, ensures mix consistency and prevents the powder from dispersing.

7.2.2 Oxidized Cellulose

These products are derived from cotton cellulose and can be provided in single or multiple sheets. There are three formulations provided for oxidized regenerated cellulose, and they come in a variety of sheet sizes. The standard form is a perforated single sheet, the fibrillar form is of a more cotton sponge-like consistency, and the more tightly knitted form comes as a thicker almost fabric-like material.

Oxidized cellulose sheets are able to create an acidic environment and are bactericidal. When conventional methods are impractical or ineffective, oxidized regenerated cellulose is indicated for control of capillary, venous, and small arterial bleeding. Beyond the mechanical effect, the cellulosic acid within the product facilitates hemostasis by denaturing blood proteins. This agent is absorbed in about 14 days. They are best used dry and can be best removed by gentle irrigation with saline. The acidic pH inactivates thrombin so the effectiveness of oxidized regenerated cellulose may not be enhanced with thrombin. Oxidized regenerated cellulose is not compatible with autoclaving or gas sterilization, thus it cannot be re-sterilized [5]. The most commonly commercial agent used in this group is Surgicel® (Ethicon), which has high tensile strength and can give excellent coverage in the presence of heavy bleeding. Two forms are available, a tightly woven knitted patch and a fibrillar form that can be used in thin layers, as tufts, or in a roll or pad. Once hemostasis has been obtained, the product should be removed, although it can be absorbed by the body [6].

7.2.3 Fibrin Glue

Fibrin glue has been considered by many surgeons as the product that comes closest to the ideal topical *glue* [7, 8]. Fibrin glue is composed of a fibrinogen concentrate (concentrate which also contains other factors active in hemostatic processes such as factor XIII, fibronectin and aprotinin) and thrombin solutions. Upon use the two components are mixed in the presence of calcium ions, thus reproducing the final stages of the coagulation process: thrombin induces detachment of fibrinopeptides A and B from the respective fibrinogen chains A-alpha and B-beta and formation of fibrin monomers which polymerize immediately by weak hydrogen bonds to form a first gelatinous and unstable clot. Factor XIII, following thrombin activation and in the presence of calcium ions, catalyzes the conversion of the weak hydrogen bonds between fibrin monomers in strong covalent bonds and consequently induces the formation of a stable, insoluble and non-friable clot. Factor XIII also induces the plasmin inhibitor (a^2 plasmin inhibitor) to bind to the clot and protect it from pre-

mature degradation by the plasmin in plasma. Factor XIII, lastly, interacts with fibronectin and other plasmatic glycoproteins to contribute to increased adhesion of the clot at the site of the lesion [8, 9]. The fibrin glue is nontoxic to the tissue on which it is applied; it promotes the formation of a clot with good adherence to the tissues in seconds; it is completely reabsorbed in a few days and it is able to stimulate the processes of tissue repair and growth of the injured tissue on which it is applied [7]. Currently different types of fibrin glue are used; they are normally divided into two categories, commercial products and those made in the laboratory (or *home-made*), which differ in the methodological preparation of fibrinogen concentrate. Fibrinogen in commercial products is extracted from a large number of plasma units, while in the home-made fibrin glue fibrinogen is obtained from individual units of allogeneic or autologous plasma mainly through a process of cryoprecipitation. Both fibrin glue preparations (trade and home-made) are widely used in various fields of surgery with the following objectives: facilitate tissue adhesion, assist surgical sutures and facilitate hemostasis [7, 9-11]. The most common clinical applications of fibrin glue are in cardiovascular surgery, thoracic surgery, and in neurosurgery. Important results have also been obtained in abdominal surgery, liver and maxillofacial surgery. The wider experience of fibrin glue is probably in the cardiovascular area where these products have been used as hemostatic agents to reduce slow and diffuse bleeding which occurs over large areas of cut surfaces or along lines of anastomosis vascular suture or needlestick sites. Several studies published in this area seem to show the effectiveness of fibrin glue in reducing bleeding in coronary bypass and congenital heart disease surgery [12]. Important results have been obtained in thoracic surgery, particularly for the treatment of bronchial injuries or fistulas leaking gas, and in resection or pulmonary decortication. In the field of neurosurgery fibrin glue has been used successfully in the treatment of dura breaches with liquor loss and as a sealant in vascular intracranial anastomosis or in dura suture after craniotomy. Fibrin glue has also been successfully used to control bleeding after dental extractions or during minor surgical procedures in patients at high bleeding risk due to congenital clotting defects or anticoagulant therapy [8, 10]. Lastly, fibrin glue has been widely used with excellent results in plastic surgery (especially to facilitate the engraftment of skin grafts in burns patients) and in maxillofacial surgery. In Europe several commercially available products are currently identified as fibrin glue, among which the most commonly used are known under their trade name: Tissucol® / Tissel®, Beriplast®, Tachosil® and Quixil®.

Tissucol®/Tissel® is a two-component fibrin glue consisting of human fibrinogen and human thrombin. Mixing the two components reproduces the latter stages of the natural coagulation cascade. It also contains fibronectin, factor XIII, plasminogen, aprotinin, grown factors (EGF, bFGF, TGFβ1, VEGF) and calcium chloride. The fibrin clot formed by tissucol has a similar structure to that of a physiological clot. It has a preparation time of ten minutes, forming a strong bond that interacts with the molecular structure of damaged tissue and renders the open tissue channels impermeable. The product provides significant adhesive force and joins elevated flaps to underlying tissues, promotes hemostasis and renders capillaries impermeable. Tissucol® is efficacious in active or capillary bleeding, in association

with topical hemostatic collagen. It also is independent of coagulation system of the patient and contains a safe inhibitor of fibrinolysis that allows the product to remain intact for 7-10 days.

Also Beriplast® is a two component plasma-derived fibrin glue composed of human fibrinogen and human thrombin. It also contains fibronectin, factor XIII, plasminogen, aprotinin, and calcium chloride. It activates fibrinogen by thrombin and is used simultaneously with specific devices. The product is adapted to promote hemostasis and sealing in all fields of surgery. Unlike Tissucol, the inconsistency of the clot formed by Beriplast, the transparency of the clot itself (which renders it more difficult to identify at the site of application and to remove it in the event of excess) and above all its dubious biocompatibility may reduce or even eliminate its regenerative power at the tissue level.

Tachosil® is a solid state fibrin glue on a support of equine collagen, with human fibrinogen and human thrombin; it also contains riboflavin, human albumin, sodium chloride, sodium citrate and L-arginine-hydrochloride. No aprotinin, factor XIII or fibronectin are present. It is indicated as a treatment support for improving hemostasis in surgery where standard techniques are insufficient. Efficacy has only been demonstrated in liver surgery.

Quixil® consists of a concentrate of human clottable proteins, tranexamic acid, human thrombin and $CaCl_2$; no aprotinin is present. It activates clottable proteins of thrombin and forms an instant fibrin clot. The product is transparent in appearance, non physiological and non elastic. It is an antifibrinolytic and is very safe thanks to the presence of high concentrations of tranexamic acid and double viral inactivation. The application site should be dry; the product is indicated to facilitate hemostasis in all surgical branches (except neurosurgery) although its effectiveness has only been demonstrated in liver surgery and orthopedics.

7.2.4 Synthetic Sealants and Adhesives

The cyanoacrylates are a group of fast-acting adhesives currently approved for topical use and not internally. Glubran® is a synthetic surgical glue based cyanoacrylate with marked hemostatic and adhesive properties which, once solidified, builds an effective antiseptic barrier against the most common infective or pathogenic agents in surgery. Glubran® is a transparent, light yellow liquid ready for use. It polymerizes rapidly upon contact with live tissue and wet environment, creating a thin elastic film with high tensile strength that ensures strong adhesion of tissues. This film conforms naturally to the tissues on which it is applied, is impermeable and is not affected by blood and organic fluids. The film, once solid, can be easily crossed by a suture needle. Polymerization time varies depending on the type of tissue with which the glue comes into contact, the nature of liquid present and the amount of product applied. Under conditions of correct application, the glue begins to solidify after about 1-2 seconds, completing its solidification reaction after around 60-90 seconds. After this reaction, the glue reaches its maximum mechani-

cal strength. Once solidified the glue has no adhesive power. Under normal surgical procedures the adhesive film is removed by hydrolytic degradation; the duration of this process varies by tissue type and the amount of glue applied. Glubran® is used in traditional surgery and laparoscopy and in digestive tract endoscopy, interventional radiology and vascular neuroradiology. It can be applied alone or in combination with sutures even in patients undergoing treatment with heparin.

Coseal® is a synthetic hydrogel composed of two ethylene glycol solutions which polymerize with a HCl buffer solution to form covalent bonds with tissues and synthetic surfaces. It seals mechanically and needs a dry surface. Coseal® is biocompatible, transparent and thus allowing the result of hemostasis to be verified, it can be sutured, it does not affect the surgery technique, it is elastic and removable, so does not damage tissues and lastly it acts as a safety barrier in the postoperative period.

7.2.5 The Future: Innovative Biotechnology for Hemostasis Control

Control of hemostasis during surgery is a problem which still has not been fully resolved. In that regard biotechnology research could suggest new methods for ensuring better hemostatic control.

One of the main lines of research in pharmaceutical biotechnology has focused on the development of formulations that can release drugs into the body at controlled quantities and rates. There are already many pharmaceutical forms by which the release may be delayed, or by which the speed and duration of the release of the active ingredient can be programmed. These formulations are therefore defined controlled-release system and they allow for the blood level of the drug to be adjusted in order to avoid an under- or overdose and minimize unwanted side effects. The result of research conducted in this area is also represented by the innovative use of special polymers as a means of releasing and managing biologically active molecules. Such polymers are capable of allowing an increase in residence time of a drug in the bloodstream or in the application, reduced antigenicity, increased stability against enzymes, and therefore greater bioavailability. These biotechnological innovations, identified as *drug delivery* systems, are not only used for systemic administration, but also for topical applications of the treatment of wounds, or the dressing of the epidermis and mucous membranes. In this case, drug delivery at the site of interest can be tracked using micro- or nanoparticles of polymeric nature as a reservoir of the drug [13]. This approach could be used for hemostasis control, as many studies on this are already present in literature [14-16].

By way of example a group of researchers at the Massachusetts Institute of Technology and the University of Hong Kong is working to develop new synthetic hemostatic agents based on self-assembling proteins. In a series of experiments on animal models, Ellis-Behnke et al. [17] have verified that these proteins, located on the wound site, organize into nanowires that form a barrier able to stop bleeding in less than 15 seconds. There are also other advantages: the protein solution used is completely nontoxic and non-allergenic and little by little is degraded, releasing

amino acids used by cells in tissue repair. Results were excellent in rats and hamsters, and similar findings are expected in humans.

As described in Sections 7.2.1-7.2.3 many commercially available products have been based on the *extracellular matrix*. These formulations are basically founded on *tissue engineering* approaches. These products have shown good clinical performance, but some improvements are still being made. The regenerated tissues resemble scar tissue and in plastic surgery this effect is better avoided. The ideal treatment would be to preserve the epidermis and promote normal collagen and elastin formation in the dermis. Regenerative medicine is a new therapeutic approach finalized at regenerating biological tissue rather than its replacement. The term *autologous bioregeneration* refers to a cell concentration from whole blood or bone marrow of the patient, obtained by physical means, which is used in situ to stimulate cell proliferation and consequent tissue regeneration. For this purpose various strategies can be used such as platelet gel or cell concentrates. Platelet gel is obtained by mixing two components: platelet concentrate to release growth factors and autologous thrombin as activator (thrombin is rich in growth factors that accelerate tissue regeneration with osteoinduction and mild hemostasis). The rationale for use is based on the fact that activated platelets release growth factors (PDGF) or express membrane molecules that favor the repair processes. Cell concentrates, however, contain several important growth factors able to stimulate various cellular mechanisms for tissue growth including: angiogenesis, macrophage chemotaxis, fibroblast proliferation and migration and collagen synthesis. Where there is significant loss of tissue, totipotent stem cell concentrates able to differentiate directly in situ can be applied. Hence, many studies are underway to ensure that wound healing occurs without the formation of scar tissue [18-20].

Hemostatic agents in previously treated fibrin glues seem to show particular potential as vehicles for drug release [21]. Several features make fibrin glues suitable for this role, such as biodegradability, due to the natural mechanism of action, the application site and biocompatibility. Some features that make the glue potential carriers of drugs, such as flexibility, high binding affinity to biological surfaces and controllable biodegradation, makes them applicable to tissue engineering, as described in the literature [22].

Given the current state of the art as presented in this chapter, the challenge lies open to provide greater support to surgical practice in an attempt to resolve the issue of hemostatic control. This challenge is being taken up by integrating our increasing knowledge of the hidden mechanisms of the coagulation cascade with knowledge of the molecular mechanisms that regulate wound repair and new developments in biotechnology.

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Gene Therapy

8

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8.1 Gene Therapy

Technological developments in gene isolation and DNA sequencing have been important factors contributing to the knowledge of the genes associated with numerous diseases. This information has been critical for enhancing our understanding of the genetic basis of disease and the role that specific genes play in human physiology.

Knowledge of data on DNA sequences has allowed the development of innovative gene therapy protocols, designed to replace or correct defective genes or insert copies of a functionally normal gene into the genome.

The goals of gene therapy are as different as:

- the correction of pathological phenotypes resulting from genetic mutations, the replacement and/or correction of a missing or defective gene;
- the expression of protein proper and permanent correction;
- the fast and efficient transfer of a *drug* while respecting the environment and cellular gene;
- the introduction into the cell of genes designed to eliminate cancer cells;
- the viral or bacterial gene transfer for the purpose of vaccination;
- the transfer of genes that stimulate growth or regeneration of damaged tissues.

The *theoretical* advantage of gene therapy is that of a radical and permanent correction of defects and the possibility of acting on the molecular mechanisms for which it is extremely difficult to develop specific drugs.

The candidate-disease (or target-disease) for gene therapy are Mendelian diseases in which the conditions or disorders arise from mutations in a single gene.

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Multigene or multifactorial disorders could be treat effectively using gene therapy, but unfortunately some of the most commonly occurring disorders, such as heart disease, high blood pressure, Alzheimer's disease, arthritis, and diabetes, are caused by the combined effects of variations in many genes, so these would be especially difficult to treat.

Other diseases might be tumors, such as leukemia or solid tumors and infectious diseases, such as AIDS.

Researchers may use one of several approaches for correcting faulty genes:

- a normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This is the most common approach;
- an abnormal gene may be swapped for a normal gene through homologous recombination;
- the abnormal gene may be repaired through selective reverse mutation, which returns the gene to its normal function;
- the regulation (the degree to which a gene is turned *on* or *of*f) of a particular gene may be altered.

Gene sequences must be delivered to the cell using a carrier, or a *vector*. A number of gene-transfer vehicles have been developed that can broadly be divided into two categories – non-viral and viral vectors. Non-viral vectors include naked DNA and DNA encapsulated with cationic lipids, known as liposomes. These vectors offer the advantages of relative ease of production and reduced toxicity. They are, however, not very effective at delivering therapeutic genes in vivo, and gene expression mediated by non-viral vectors is often transient.

The viral vectors most commonly used in gene therapy are viruses that have been genetically altered to carry normal human DNA. Viruses have a unique ability to recognize certain cells and insert genetic material into them. Given the need, in many diseases, for sustained and often high-level expression of the transgene, attention has turned to viral vectors. Viruses have the ability to gain access to specific cells and exploit the host's cellular machinery to facilitate their replication. Recombinant viral vectors are designed to harness the viral infection pathway. However, they lack the ability to replicate in the host cell, as the coding sequences for viral replication are replaced with the gene of interest, such as β-globin or human factor IX for thalassemia of hemophilia B, respectively. The number of different viruses that are under development as gene-therapy vectors is steadily increasing, but they can be divided into two general categories – integrating and non-integrating. At present, retroviral vectors based on onco-retro or lentivirus are the only gene-transfer systems that can mediate efficient integration of the transgene into recipient cells. The latter are particularly advantageous in that they can infect both dividing and quiescent cells and confer stable transgene expression for a long time. Despite their advantages, safety concerns have been an obstacle to the application of lentiviral vectors in human trials. In contrast, the genome of vectors based on herpes, adeno-associated or adenovirus vectors is mainly maintained as episomes. They do not usually integrate into the host genome and are consequently lost over time. Therefore, expression from non-integrating vectors is often transient, especially in tissues/organs with a high cellular turnover. This may be ideal for a

number of cardiovascular and malignant disorders where only transient expression is required, but may necessitate repeated vector administration if used for the treatment of chronic disorders [1].

Non-viral vectors include microinjection and/or electroporation, for inserting DNA directly into the nucleus.

The technology of gene targeting through the process of homologous recombination is an approach focused on modifying a specific sequence *target* of the cellular genome. Gene targeting induces a site-specific chromosomal modification that leads to long-term and genetically inheritable expression of the correct gene, regardless of its size. Moreover, direct conversion of genomic sequences preserves the integrity of the gene in such a way that the coding sequences and regulatory elements remain intact. The availability of better delivery methods has made non-viral transfer an increasingly safer and more viable method for gene therapy [2]. Among these, human artificial chromosomes (HACs) are highly promising vectors recently optimised in gene therapy applications [3]. They are able to maintain expression of genomic-sized exogenous transgenes within target cells, without integrating into the host genome. Although these vectors have huge potential and benefits when compared with normal expression constructs, they are highly complex, technically challenging to construct and difficult to deliver to target cells. HACs contain the capacity for large gene inserts, carrying genomic loci also with regulatory elements, thus allowing for the expression of transgenes in a genetic environment similar to the chromosome.

Another important key to the success of gene therapy is the target cell. Embryonic or fetal stem cells are, for example, significantly more versatile and plastic than their adult counterparts.

Stem cells are defined as cells that have the ability to renew themselves continuously and possess a pluripotent ability to differentiate into many cell types. Different types of mammalian pluripotent stem cells, embryonic stem (ES) cells derived from the inner cell mass of blastocysts and embryonic germ (EG) cells obtained from post-implantation embryos, have been characterized. Also tissue specific stem cells could be isolated from various tissues of more advanced developmental stages such as bone marrow mesenchymal stem cells, hematopoietic stem cells and neural stem cells, and also extra-embryonic foetal tissue like chorionic villus and amniotic fluid [4, 5]. However, the clinical applications of human embryonic stem cells are limited by ethical concerns, as well as the potential for teratoma formation. As a consequence, alternative forms of stem cell therapies, such as induced pluripotent stem cells (iPS) and bone marrow-derived mesenchymal stem cells, have become an area of intense study [6, 7]. To date, iPS cells resemble pluripotent stem cells in all characteristics tested. These cells are similar to ES cells in morphology, proliferation, telomerase activity, gene expression, epigenetic status and at the same time are able to differentiate into the three germ layer lineages and to give rise to adult chimeras. The power of induced pluripotent stem (iPS) cells is that they do not require the destruction of embryos and they may one day be produced from the patient's own adult cells, allowing the generation of perfectly matched tissues for transplantation therapies. Obtaining patient-specific pluripotent

stem cells from adult differentiated somatic cells is a new exciting strategy that has gained great impetus recently. When there is a genetic disease, iPS cells have the same deficit and so gene therapy protocols have to be developed to correct them before re-implanting back into the patient. Given the immense regenerative potential of these pluripotent stem cells, it will be imperative to understand each milestone in the differentiation process for a particular organ or tissue type in order to use them in stem cell therapy.

However, the translation of human pluripotent stem cells into cell/gene therapies will require the development of standardized tests for product consistency, reproducible differentiation, stability, tumorigenicity, toxicity and immunogenicity.

After transplantation in combined cell/gene therapy approach, stem cells can provoke the multiplication and function of the host stem cells, prevent further loss and damage of host tissues, or stimulate the production of new blood vessels that can help to restore host tissues. The transplanted cells presumably produce these effects by producing growth factors and other products that are locally active. Transplanted stem cells can also fuse with host cells, and this can help repair damage even if the transplanted cells are not completely transformed into the target tissue type.

The pluripotency of the cells allows generation of a wide array of differentiated cell products, but it also entails the possibility of teratoma formation and the presence of unwanted cell types. The tumorigenic potential of hESC-derived products may vary dramatically depending on cellular composition and host environment. If feasible, implantation of the cell products should be performed in the context of the appropriate disease and immunosuppressive therapy [8]. The determination of the safety of any pluripotent stem cell product is inseparable from an assessment of the risk/benefit ratio in each clinical indication. As with any other therapy, it is wise to acknowledge that it is generally not possible to eliminate all risk.

Stem cells have considerable utility as targets for gene therapy because they can self renew, thus precluding the need for repeated administration of the gene vector. While this approach has already been validated in post-natal clinical trials with HSCs [9-11], the occurrence of leukemia in three of 11 children treated in the French trial and in one of ten children from the London trial [12] due to the insertion of the transgene near a proto-oncogene has raised serious concerns about the risk of insertional oncogenesis. Several lines of investigation are currently being pursued to reduce this risk, such as the use of insertional site screening prior to re-infusion, the use of safer and/or site specific vectors and the use of adjustable vectors, which could be switched off, if an adverse event arises [13].

Despite an abundance of promising studies in animal models, the translation of preclinical experiments into clinical trials has been minimal. The success of gene therapies has been limited because of persistent challenges relating to the choice of gene transfer vehicle, efficiency of gene transfer, level of therapeutic gene expression and potential for unwanted immunological responses against the vector or the transferred gene. Nonetheless, promising clinical trials using gene therapy have been completed, and many more trials are underway. Recent examples of clinical success in this area include the long-term engraftment of retrovirally transduced

hematopoietic stem cells into patients with SCID [14], and the application of gene therapy to patients with Leber's congenital amaurosis (an inherited eye condition) [15]. The development of novel gene therapy vectors with reduced immunogenicity or toxicity will also be important to move forward the application of this research to potential clinical applications for non-life-threatening diseases.

Currently, cell therapy protocols are regulated under the US Food and Drug Administration's (FDA's) Good Tissue Practices Final Rule. Although there are no approved stem cell products, several are in late-stage clinical trials. Updated and thorough information on gene therapy trials, showing the number of approved, ongoing or complete clinical trials worldwide, is available on the web at http://www.wiley.co.uk/genetherapy/clinical/.

Lastly, combined cell/gene therapy has the potential to move toward multiple clinical applications within the next few years, and, in addition, is likely to be increasingly valuable as a tool for investigating the mechanisms of acute and chronic allograft loss in more detail.

8.2 Gene Therapy for Neurodegenerative Diseases

There are two broad categories of gene therapy: (1) *ex vivo* transplantation/implantation of cells genetically modified in the laboratory; and (2) in vivo direct insertion of a gene into the patient's own cells. Several in vivo approaches to gene therapy for neurodegenerative diseases are currently being pursued both in animal models and in early human clinical trials.

In this section different and novel approaches of both in vitro and in vivo gene therapy for acute and chronic neurodegenerative disorders affecting neurons and glial cells in the brain and spinal cord will be reviewed.

Specifically we will focus our attention on neuromuscular disorders (NMDs), such as Duchenne Muscular Dystrophy (DMD), myotonic dystrophy (MD) and spinal muscular atrophy (SMA), characterized by loss of ambulation, chronic disability, and early death worldwide. Motor neuron diseases (MND) show a progressive degeneration of upper and lower motor neurons. The most common forms include amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA) and spinal bulbar muscular atrophy (SBMA). These disorders have genetic causes because of mutations in different genes that lead to a variety of phenotypes that differ in severity and/or target organ. Current therapies are largely palliative and delayed and are unable to prevent the progressive loss of muscle and/or function and increasing disability.

Research into therapeutic approaches for both recessive and dominant neuromuscular disorders has made great progress over the past few years. Replacing the defective gene is unlikely to be therapeutic at all. It is therefore not surprising that for dominant neuromuscular disorders, attention has focused on ways to restore gene expression at (pre-) mRNA level. This can be achieved through antisense

oligonucleotides (AONs), small synthetic RNAs, DNAs or analogs, which hybridize specifically on their target sequence [16].

Myotonic dystrophy (MD) is an autosomal dominant disorder in which an interfering gene product (*DMPK* mutant) is responsible for the disease [17]. The mutant *DMPK* gene produces an mRNA that contains an expanded (CUG)n repeat in the 3'-untranslated region. The expanded repeat (N>37) causes a disruption in global pre-mRNA splicing resulting in expression of neonatal, rather than adult isoforms from a variety of genes.

DMD is an X-linked recessive disorder that affects 1/3500 males caused by the loss of dystrophin, a protein essential for maintenance of muscle fiber integrity. AONs can be used to reframe dystrophin transcript, by hiding an exon from the splicing machinery and causing the exon skipping [16]. This will allow the generation of a partly functional dystrophin protein causing Becker muscolar dystrophy (BMD), thus converting a severe phenotype into a milder one.

Some of the most promising experimental therapies currently under investigation for the treatment of DMD focus on the restoration of dystrophin expression in myofibers. Numerous strategies have emphasized either the introduction of a functional, recombinant version of the *dystrophin* gene or modification of dystrophin pre-mRNA splicing. Modification of dystrophin splicing via AOs was initially demonstrated in a cell-free system [18] and soon progressed to evaluation in human myoblasts. Through extensive analysis of AO targets, it has become apparent that the skipping of each exon, or group of exons, will need to be evaluated on a case-by-case basis. For example, skipping of exon 23 in the dystrophin-deficient *mdx* mouse has been highly successful [19], demonstrating the restoration of dystrophin expression in the *mdx* mouse mediated by antisense oligonucleotides conjugated to an arginine-rich cell-penetrating peptide. This is the first study to achieve successful cardiac dystrophin expression following systemic injection of morpholino antisense oligonucleotides. Questions remain about host immune system toleration of the added peptide. Despite these challenges, research is rapidly progressing to evaluate the feasibility of exon skipping in vivo.

Another attractive strategy for these disorders is a gene targeting approach by which a mutated gene is converted to one with the normal (or desired) sequence [20]. Small fragment homologous replacement (SFHR) is a site-specific gene modification approach that has the potential to maintain the genomic organization necessary for expression. This method has been examined for mutations of the *dystrophin* [21] genes, and partial gene corrections were obtained. This study investigates the in vitro and in vivo application of a double-stranded method variant of SFHR gene repair to the *mdx* mouse model of DMD. A 603-bp wild-type PCR product was used to repair the exon 23 C-to-T *mdx* nonsense transition at *dys* locus in cultured myoblasts and in tibialis anterior from male *mdx* mice. Conversion was observed at both the DNA and RNA levels. The conversion of *mdx* to wild-type sequence in vitro was about 15 % by PCR analysis, although there was no detection of normal dystrophin protein. The correction efficiency in vivo was up to 0.1 % in the tibialis anterior of male mdx mice, but again there was no evidence of gene expression at either the transcript or protein level. Recently, the first development

of a HAC vector containing the entire human *dystrophin* gene (DYS-HAC) has been shown that is stably maintained in mice and in human immortalized mesenchymal stem cells (hiMSCs) [22]. This kind of vector is very useful for transferring large sequences together with their regulatory elements, such as *dystrophin* gene.

The SFHR approach was recently used for another genetic disease, spinal muscular atrophy (SMA) [23], caused by *SMN1* gene loss in humans. The presence of the *SMN2* gene, the ortholog of *SMN1* gene in humans, and the capacity that *SMN2* has in rescuing embryonic lethality in SMN-deficient mice has illustrated the importance of SMN protein for cell survival [24]. Thus, the replacement of *SMN2* gene using a gene therapy approach to treat SMA patients appears to be an attractive therapeutic strategy. A gene targeting approach such as SFHR has been successfully used [23] in vitro in human chorionic villi (CVS) derived from SMA fetuses allowing an increase of up to 53% in full-length SMN mRNA compared with untransfected controls. Furthermore, genotype and phenotype of transfected cells remained stable after several in vitro passages, demonstrating the stability of the correction over time [23].

AAV-mediated delivery of other therapeutic transgenes has also been explored for SMA and ALS. Thus far, no substantial therapeutic treatment exists for these disorders, but hopes could come from progress in gene-based and cell-based therapies.

Azzouz et al. replaced the defective gene in a mouse model of SMA showing beneficial effects following intramuscular injection of a lentiviral vector. However, only a modest increase in life span was demonstrated (3 to 5 days) when SMA mice were injected with lentiviral vectors expressing *SMN* gene [25]. This study shows that this approach is attractive and a better therapeutic result might arise if the transduction was more widespread (restore SMN expression in cell types other than only motor neurons) and/or if the administration took place at an earlier stage (e.g. *in utero*) in order to allow to the exogenous SMN protein, expressed by the vector, to compensate the loss of the endogenous one.

More recently independent groups published two interesting paper [26, 27] in which an altered virus (scAAV9-SMN) was used to deliver *SMN1* into the facial veins of newborn SMA mice ranging in age from 1 to 10 days old. The SMN-laced viral vector injected into the P1 mice reached almost half of their motor neurons, resulting in improved muscle coordination, properly working electrical signals to the muscles and longer survival than in untreated mice. This particular viral vector can cross the blood-brain barrier, a characteristic that is required to ensure that the scAAV9-SMN vector can reach nerve cells in the spinal cord. Ten days after the injection, about 42% of spinal motor neurons contained the fluorescent protein. Similarly, SMA mice receiving the SMN viral vector at one day old showed a protein increase in the brain, spinal cord and muscles within 10 days, even though the levels remained lower than the levels of *SMN* in normal mice. This study hopes to progress to human clinical trials as soon as the requisite toxicology experiments are in place and federal regulators will allow.

Utilizing the vectors described earlier, new therapies for neurodegenerative disorders are being developed. As with standard medical therapies, there have been a number of approaches: neuroprotective/restorative therapies, aimed at slowing or

reversing disease progression. Strategies utilizing genes coding for nerve growth factors may be applied to all neurodegenerative disorders, but disease-specific strategies are being developed as well. Several neurotrophic factors have shown promise as therapeutic agents in animal models of neurodegenerative disorders. Gene therapy with glial cell-line derived neurotrophic factor (GDNF) has shown to be promising, for example in animal models of ALS. Regarding ALS disease an in vivo strategy was reported by delivering GDNF, insulin-like growth factor-1 (IGF-1) or vascular endothelial growth factor (VEGF). This treatment showed to be effective in preserving motor neurons in ALS mice and increasing their survival [28-31]. GDNF appears primarily to influence disease onset and not progression [29-31], making it an unlikely candidate for gene therapy in ALS, while IGF-1 and VEGF both demonstrated to be efficacious when delivered after clinical onset of the disease (50% motor neuron loss), making them effective in potential clinical trials [30, 31]. Although viral delivery of growth factor, appears to hold promise for future use in patients with MND, careful considerations about delivery risk have to be evaluated. First of all, the virus could be transported to other regions of the central nervous system (CNS), potentially causing side effects [32]. Secondly, virus-integration in proximity to oncogenes could trigger malignant cell proliferation [33]. However the use of adeno-associated virus or lenti-viral vector is unlikely to cause toxicity [34], because both vectors show capacity for retrograde transport from the muscle to the spinal cord [30, 31, 35]. Undoubtedly the use of cellular transplants to deliver growth factors could provide a safer method of delivery. For these purposes, embryonic (ES) and neural stem cells (NSCs), and more recently induced pluripotent stem (iPS) [36] are potential cellular sources which could be harnessed in the treatment of numerous disorders. Several cell-based approaches for the therapy of motor neuron disorders have been reported with the aim of creating trophic support to preserve endogenous cells and for host cells replacement [37, 38]. The selective degenerations of motor neurons in discrete regions of the brain and spinal cord, characteristic of ALS, SMA and SBMA diseases, strongly encourage researchers to experiment cell replacement strategies, by using stem-cell derived motor neurons [39]. Towards this goal, mouse ES cell-derived motor neurons were recently used in a rat model of virus-mediated acute motor neuron death [40]. More recently, murine neural stem cells (NSCs) were also utilized for transplantation into presymptomatic *nmd* mice [41] and SOD ^{G93A} mice [42]. NSC populations were isolated from either embryonic spinal cord and enriched by cell sorting (FACS) based on their high aldehyde dehydrogenase (ALDH) expression or from adult brain and purified based on Lewis X and the chemokine receptor CXCR4-expression [42]. In both studies, the animals showed a 3-week delay in onset, correlated with a partial preservation of host spinal motor neuron, and also a 3-week increase in mean survival time. The beneficial effects of grafted cells on host motor neuron survival and disease onset appeared to be through trophic support, based on graft release of IGF-1 and VEGF.

In other study, Corti et al. isolated spinal cord NSCs from SMA mice expressing green fluorescent protein only in motor neurons and assessed their therapeutic effects on the phenotype of SMA mice [43]. Intratechally grafted NSCs migrated into the parenchyma and generated a small proportion of motor neurons. Treated

SMA mice exhibited improved neuromuscular function, increased life span, and improved motor unit pathology. NSC transplantation positively affected SMA phenotype, indicating that transplantation of NSCs may be a possible treatment. However, most potential sources of cell therapeutics, such as fetal-derived primary neurons, human ES cells, and endogenous neural stem cells, are associated with ethical controversy or present technical limitations. The subsequent successful generation of human iPS cells from skin fibroblasts opened the door for iPS cell based therapies. To this end, Dimos et al. [44] obtained fibroblast lines from two elderly siblings with early or late manifestations of ALS, and Ebert et al. took a similar approach from SMA skin fibroblasts patients [45]. Therefore, the combination of patient-specific iPS cells and HAC-containing defective genes represents a powerful tool for gene and cell therapies.

The generation of iPS cells from a patient's own skin fibroblasts would potentially allow auto-transplantation, with the elimination of immune rejection problems.

Despite several successful results, additional basic research is required to effectively understand and control unknown mechanisms regulating the proliferation, migration, differentiation, survival, and function of stem cells and stem cell-derivatives to be used. In this way the clinical harnessing of stem cells to treat neurodegenerative diseases could be guaranteed.

8.3 Local Gene Delivery for Tissue Repair in Surgery

V. Lionetti

8.3.1 Introduction

In the past two decades, advances in cellular and molecular biology have expanded our understanding of tissue repair and regeneration [46]. Tissue repair encompasses a complex process that requires the integration of cell rescue as well as extracellular matrix turnover, angiogenesis, and remodeling under genetic and epigenetic control [47]. Recent progress in basic [48] and clinical research [49] revived tissue repair based on gene therapy as an attractive option for efficient and targeted treatment of a wide range of inherited and acquired complex diseases, whose only therapeutic option is the surgical approach or palliative long term drug treatment. It has now been established that several crucial issues, such as gene delivery and molecular mechanisms of tissue repair, are still to be resolved prior to achieving clinical success in regenerative medicine. The concept of *gene-based tissue repair* includes the use of transgenes encoding several peptides that modulate the secretory and life-saving capacity of resident cells. The targeted delivery of transgenes for local regenerative purposes, compared with pharmacological treatment, has shown several advantages: (a) targeted expression of specific trophic factors in the native form [50], (b) sustained and efficient modulation of intracellular target signaling activa-

tion related to the trophic factor tissue availability [51], and (c) limitations of systemic side effects. Therapeutic genes can be delivered in vivo as naked or packaged into viral or non viral vectors. In vivo techniques are based upon the direct introduction of genes to the target tissue. The selection of an appropriate vector for the transfer of genes is critical for overcoming the physiological barriers and is paramount for success of gene-based tissue repair [52].

8.3.2 Barriers to the Delivery of Genes for Tissue Repair In Vivo

To mediate gene-based tissue repair, intact and active oligonucleotides have to be introduced into the cytoplasm and efficiently integrated into the nuclear DNA of target cells. The cellular uptake is the main barrier for the delivery of nucleic acids, which limits even the local transfer. In addition, many tissues in vivo may only be reached through the systemic administration of genes via the blood stream. For this purpose, to guarantee effective and non toxic systemic delivery, the genes have to remain intact in the bloodstream, extravasate through the vessels, diffuse through the extracellular matrix (ECM), pass the cellular membranes and be released into the cytoplasm. Naked circulating nucleotides have a very short half-life, owing to degradation by circulating nucleases, rapid renal excretion, aggregation with serum proteins and phagocytosis by immunocompetent cells. Some of these issues, such as plasmid degradation and inactivation, can be overcome by introducing chemical modifications or assembling plasmid with proteins, lipids or polymers, which allow resistance against nuclease degradation [53], or by packaging nucleotides in viral vectors [54]. Even if they survive in the plasma, the next major barrier of gene delivery is the tight vascular endothelial wall. Generally, molecules larger than 5 nm in diameter do not readily cross the capillary endothelium [55]. Alternatively, vectors could be locally injected in the damaged tissue by electroporation or gene gun, or assembled into tissue engineering scaffolds and gradually released at the injury site to overcome the endothelial barrier [56]. However, after the circulating gene delivery complex passes through the vasculature, it must diffuse through the ECM, which is a dense network of polysaccharides and fibrous proteins that can hinder nucleotides complex diffusion and limit efficiency of gene delivery in vivo. The intracellular barriers included cellular internalization, endosomal escape, vector unpacking, and transport into the nucleus. The fundamentals underlying viral and non-viral delivery system, and the more recent development of polymeric delivery systems are described below.

8.3.3 Viral Vectors

Gene transfer using viral vectors relies on the efficient ability of viruses to carry and express their genes into resident cells to enhance tissue repair [57]. Each viral

vector is developed by deleting the native viral genome and inserting the desired therapeutic gene. Human adenoviruses (Adv) are double-stranded DNA viruses and the most common vectors used in viral gene therapy allowing a stable and transient gene expression in non dividing cells. Unfortunately, Adv are highly immunogenic, probably due to the capsid proteins [58].

However, adeno-associated viruses (AAV) are single-stranded, non-enveloped DNA viruses [54]. AAV transduce a variety of cells, including non dividing cells, provide long-term gene expression, and are less immunogenic. New experimental approaches with AAV indicate that different serotypes might influence the targeting abilities and transduction rates of specific cells, such as endothelial cells, to promote gene expression and therapeutic angiogenesis [59].

Retroviruses (RV) are single-stranded RNA viruses that replicate through a DNA intermediate and can be designed as highly tissue specific and safe vectors from recombination after transfection [60]. They offer the potential for long-term gene expression through integration into the host cell genome. However, RV are not able to transduce non-dividing cells. Conversely, lentiviral vectors are able to make non-dividing cells a target for long-term gene expression [61]. Unfortunately, the ability of viral delivery to promote tissue repair is limited by the induced immune response, which might be in part controlled by polymeric encapsulation of the viral vector. In fact, polymeric encapsulation shields the vector from neutralizing antibodies, reduces the quantities of vector required in vivo and serves to locally concentrate the virus in an efficient dose [62].

8.3.4 Non-viral Vectors

Non-viral vectors are less toxic and immunogenic than viral vectors, yet the gene delivery efficiency might be significantly lower. Several non-viral carriers developed to improve in vivo localized gene transfer, proved effective in enhancing tissue repair. For this purpose, oligonucleotides might be linked to synthetic molecules, such as cationic polymers (polyethyleneimine, chitosan) [63], peptides (HIV-TAT) [64], liposomes (PEGylated lypsomes, stable nucleic acid-lipid particles) [65] or aptamers (small synthetic highly structured nucleic acid molecules) [66]. These DNA-nanoparticles come through the cells by endocytosis, yet the low capacity of intracellular esocytosis is considered the major limitation of their transfection efficiency. Non-viral vectors/DNA repairing complex might be directly injected into the damaged tissue, systemically injected under guidance of noninvasive imaging (ultrasound, magnetic resonance imaging) or immobilized to biomaterial substrates (coils, stents, membranes) by drying nonspecific adsorption or retained within biodegradable scaffolds (hydrogels) to provide gradual release.

8.3.5 Tissue Engineering Scaffolds

Viral or non-viral vector-loaded scaffolds can stimulate tissue formation and could be directly implanted into an injury site for delivery to cells within or near the scaffold [67]. Scaffolds can distribute the gene material throughout the microenvironment of damaged tissue, and have proven more effective than single injection. These innovative biodegradable biomaterials are categorized according to the basic delivery mechanisms:

- polymeric release: sustained oligonucleotides delivery from polymer substrates (collagen, collagen-gelatin, polylactic glycolic acid, polyethyleneimine) to enhance efficiency of gene transfer by maintaining elevated, and not cytotoxic, tissue levels of nucleotides and allowing repeated cellular uptake;
- substrate-mediated delivery: immobilization of gene complexes within biomaterials which serve as substrate for cell adhesion (i.e. biotin cationic polymers binding neutravidin substrate, collagen membranes, hyaluronic acid-collagen hydrogels). DNA is directly placed in the cell microenvironment to facilitate cell internalization and allow spatially controlled gene delivery.

The mechanical properties of the scaffold and mechanical stimulation of the tissue can influence tissue formation activating expression of genes related to tissue repair [68].

8.3.6 Conclusions

The relationship between gene delivery, transgene expression and tissue repair/replacement remains a significant challenge in design and choosing innovative tools for in vivo use of gene therapy in regenerative medicine. Although the recent description of multiple targeted delivery systems heralds future therapeutic applications, there are still a number of concerns and scope for improvement. The potential risk of toxicities and lethal immune responses to the vehicle component as well as to the targeting component needs more thorough evaluation. In this regard, peptide-based systems, non toxic liposomes and less immunogenic viral vectors might be clinically relevant in the future. The use of biodegradable scaffolds in gene delivery can better modulate local transgene expression and guarantee a remarkable increase of the duration of transgene expression than the duration of release within the context of the signals present in the microenvironment. In conclusion, the exciting technological advances in gene delivery here point to a bright future for gene-based tissue repair in surgery.

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Stem Cells

9

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9.1
Philosophical and Lexical Issues

To fully understand the biological meaning of the term *stem cell* (SC) it is useful to clarify the derivation of the root *staminal*, even though modern research published in English-speaking journals never seem to use the term staminal. While there are no doubts that the term SC originated in the context of two major embryological questions, the continuity of the germ-plasm and the origin of the hematopoietic system [1], it is not clear at all which is its etymological derivation. This is a relevant point for the consequences and the impacts that SC biology has in society, particularly on the beliefs of lay-people (i.e. decision makers) who so frequently impact on research freedom. We would like to suggest that the term is in fact a neo-Latin word (the adjective **staminalis*, -e never existed in Latin!) that was coined in an English-speaking scientific environment. In any case, the word originates from the Latin *stamen*, -*inis*, a neutral noun that is formed by two distinct morphemes: the lexical base (of Indo-European origin) *sta- *to be firmly placed*; *to remain*; and the derivational morpheme (of Indo-European origin) *-men-, which is widely used to form nouns that indicate firstly the production of the effect of an action, and hence, by implication, the effect of the action itself. Some examples of the -men morpheme in Latin include: ag-men *army on the march*; lu-men *the glare of the light* (vs. lux *absolute/real light*); ful-men, *the flash that precedes thunder*; cri-men, *the action of separating/selecting and so figuratively, the object of selection*; se-men, in the first place, *the action of sowing*, and consequently *that which is sown*.

Stamen was, originally, a technical weaving term indicating the warp of the cloth (literally *that which is firmly fixed*), onto which the weft is woven in order to

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form the final product. More generally, it indicates *the thread* and, metaphorically, *the fiber of life; destiny; fate; the thread of life*. What is important to note in the semantic value of the Latin word *stamen*, is the idea (abstract) of something that *remains firm, stable*, which serves as a *basic, founding principle*. Hence, basic, founding *stem cell*. However, our original explanation of the term SC is not the only one. Recently, Ramalho-Santos and Willenbring [1] suggested that: "One would be tempted to assume that the term stem cell has some relation to the term *meristem*, because meristems are the stem cell compartments of plants. The term meristem was first used by the Swiss botanist Karl Nägeli in 1858 to refer to the areas of continuing cell division in a plant (Nägeli, 1858). Nägeli derived the term meristem from the Greek *meristos* (divided/divisible) and the suffix *-em* (as in *phloem* or *xylem*). Hence, the terms stem cell and meristem, despite being similar and having overlapping meanings, were developed independently and are etymologically unrelated."

Regardless of the etymological derivation of the term, the SC is defined both as a cell able to divide itself in such a way that it originates a daughter cell identical to the mother cell (renewal capacity) and as a cell able to differentiate itself into a specific cell type of one of the many tissues that make up the animal body (differentiating capacity). Therefore, it is a cell endowed with a double capability: renewal and differentiation, two elements fundamental to the stable and steady physiological maintenance of an organism. This extraordinary biological duality interests biologists who are trying to discover the molecular mechanisms at the basis of embryonic development, cell differentiation and the genetic reprogramming of differentiated cells. Furthermore, it is of interest to the medical community in the conception of technical applications for the development of cell replacement therapies in tissue that has been damaged as a result of aging, trauma or of specific diseases.

Until the beginning of the 1950s the existence of SC was mostly conjecture and based on the physiological concept of the *dynamic state of body constituents*. This concept dates back to the 5th century BC; it was recorded by the philosopher-naturalists long before Aristotle; in particular, Heraclitus (540-475 BC) records and uses it frequently. The existence of cells reputed to renew tissue is intuitive (think about skin and blood!); it follows, likewise intuitively, that these initially putative cells would enable the complete renewal of the body and that, accordingly, they would be involved in the longevity of an organism. All these intuitions return overwhelmingly as well established fact in our current knowledge of the role of SCs in the development of many diseases (e.g. cancer) and more generally in the projected development of cell-based therapies (regenerative medicine). In 2006, the European Molecular Biology Organization (EMBO) published a summary document titled Stem Cell Research - Status, Prospects, Prerequisites, which can be downloaded for free (http://www.embo.org/index.php), introducing the field of SC biology. The reader is strongly advised to refer to this still valid document which outlines all of the relevant problems related to one of the hottest topic in biology and medicine today.

9.2 Origin, Animal and Vegetal Models

SCs can be derived from various sources; from the early stages of embryonic development, before implantation in the uterus (embryonic stem cells, ES), and from the post-implantation and post-natal development stages (somatic or adult stem cells, SSC). SCs are classified on the basis of their differentiation potential, a potential that becomes increasingly limited as development progresses from the embryonic and post-natal stages through to senescence. The *totipotent* SC is the single-cell embryo, the zygote, which is formed by the union of the sperm and the egg cell. Through embryonic and fetal development, the zygote forms a new organism that at the adult stage (in humans) consists of about one million billion cells (10^{15}). All these cells originate from the zygote, and it is on account of this generative ability that the zygote is called *totipotent*. The zygote and the embryo cells (blastomeres) in the early stages of pre-implantation development (blastocysts) possess all the information, at a nuclear and cytoplasmic level, needed to produce the different types of cells that make up a new individual. Changes in the number and in the type of genes that are expressed in every stage of development lead initially to the determination of the differentiation fate of the cells, and subsequently to their actual differentiation into the different types of cells that are present in the adult organism. The cells that make up the early stages of embryonic development (the blastocysts) are called *pluripotent* because they are able to generate the cells that differentiate themselves into the three embryonic layers, the endoderm, the mesoderm and the ectoderm, which in turn will create all the cell types that make up the various tissues and organs. *Multipotent* and *unipotent* stem cells are those that have a limited differentiation ability and are found in the later stages of fetal development and in the adult. These stem cells exclusively generate the cell type of the tissue in which they are found: again, picture the self-renewal that characterizes blood and skin stem cells.

The list of tissues and organs from which it is possible to obtain SCs is growing by the day (recently SCs have been found in the pulp of deciduous *baby* teeth, as well as in the nervous tissue of cadavers 18 hours after death). There are therefore numerous sources for SCs, including of course the umbilical cord and material derived from spontaneous and induced abortions. Additionally, cloning techniques have very recently led to the development of methods that enable the derivation of SCs from terminally differentiated cells (somatic cell nuclear transfer technique, the use of cytoplasts, and retroviral transfection of SC genes) as will be shown later.

Regardless of its origin, a SC is defined by its intrinsic capacity for self-renewal. There are several hypotheses accounting for the SC self-renewal capacity, the most favored being the asymmetric hypothesis which states that one of the daughter cells keeps the peculiar stemness property of SCs while the other cells are committed to differentiation. Several molecular mechanisms have been put forward to explain the stemness property based on the asymmetric mitotic division, but two of them are much popular than others: the immortal strand hypothesis advanced by

Cairns in 1975 [2-4] and the hub-niche hypothesis put proposed by Schofield in 1978 [5]. The first suggests that after DNA replication the segregation of the strands follows a pattern by which the oldest *template* strands are continuously retained by one of the *new* generated cells (that will become a SC) while the other new daughter cell will inherit all of the younger new template strands (and will be committed to differentiate). This hypothesis is quite fascinating since it can account for the origin of cancer cells, namely for the origin of cancer stem cells (CSC), since the replication-induced mutations are all inherited by just one of the two new daughter cells, the non-SC. As time passes, mutations accumulate in only one cell (in humans at an average rate of one mutation per seven mitotic divisions, roughly a mutation rate of something like 10^{-5} – 10^{-7} per gene per generation) thus easily reaching a critical threshold which triggers an inflammatory (intermediate) state from which, as mutations accumulate, the neoplastic (stemness) condition arises due to the deregulation of the tumor suppressor genes activities [4, 6]. A clear datum supporting this view comes from the demonstration by Reya and Clevers [7] that the Wnt signaling deregulation phenomenon is the common and precocious event in malignant progression of colorectal, breast, skin and hematopoietic tumors. The Wnt deregulation likely acts by activating the SC self-renewal within the SC's niche. An inclusive view of the CSC field is presented and continuously updated by Riccardo Fodde, leader of the *MCSCs* European group (the "Migrating Cancer Stem Cells" consortium: www.mcscs.eu).

The second hypothesis, known as the SC niche hypothesis, states that SC are maintained in a very specialized anatomical compartment where both proximal (cell surface molecules) and distal (secreted molecules) signals provide a definite microenvironment (the niche) controlling cell proliferation and differentiation, thus protecting the SC from exhaustion. This concept came out of seminal studies on the hematopoietic system [5], the *Caenorhabditis elegans* germ cell development [8], the *Drosophila melanogaster* ovary [9, 10] and some model plant systems like *Arabidopsis thaliana* (see the 2007 review by Scheres on SC niches across kingdoms [11]). The SC niche is nowadays a paradigmatic concept in SC biology [12], even when its role is critically considered [13].

9.3 Somatic and Embryonic Stem Cells

A chronology of SC researches can facilitate comprehension, above all of the scientific problems, but also of the ethical and legal problems linked to the derivation and use of such cells. They were first proven to exist by Canadian researchers at McGill University (Montreal, Quebec, Canada) in the 1950s. This milestone was made possible thanks to the development of several histological techniques, which in turn were linked to advances in the fields of nuclear physics and radioactive isotopes. While cells and tissues were initially studied in terms of their 3-dimensional architectural relationships, the development in the 1930s and 1940s of autoradiography

techniques added a fourth dimension: that of time. Accordingly, Charles Leblond and his colleagues (H. Cheng, W. Chang, J. Marques Pereira, B. Messier, J. Nadler) demonstrate the dynamic of synthesis, and the movement between different types of cells, of substances (sugars, aminoacids) that had always occurred in cells but that were now traced and stained with chemical elements capable of emitting electrons (for example, radioactive isotopes of hydrogen, sulphur, and carbonium).

They were thus able to show that the cells at the base of intestinal villi are capable of dividing themselves asymmetrically, as had been hypothesized, and so of renewing the epithelium that covers the villi: clearly, these cells were, and are, SCs. Leblond proved the existence of SCs in the testicle by identifying a particular type of cell, called Spermatogonium A, which, with the production of sperm, is capable of ensuring the constant renewal of the seminiferous epithelium. The two researchers who in the early 1960s established several tests which are still in use today to define the nature of the SC were also Canadian: Ernest A. McCulloch and James E. Till launched the field of SC research with a study that was to become the cornerstone of SC biology. This study regarded hematopoietic SCs, the tissue "study model" that is able to provide many of the conceptual paradigms that we use today for the molecular dissection of the stemness. Till and McCulloch developed tests to measure the differentiation of SCs (in vitro) and their ability to repopulate a tissue (in vivo). Of particular note are the clonogenicity test and the serial transplant test, both still essential for SC researchers. The Canadians' groundwork was followed by a series of contributions from the scientific community at large which showed the existence of SCs in all the different anatomical compartments. Thereafter, and as is typical of scientific endeavor, there was a succession of many small advances in knowledge; though individually slow, collectively these advances rapidly enabled the use of SCs in numerous therapeutic applications. Some of these applications are already well consolidated (bone marrow transplants, artificial skin, cornea replacement), while others are under trial (e.g. Parkinson's Disease, heart infarction and diabetes), and yet others are still at the experimental stage (spinal stroke, Alzheimer's disease, amyotrophic sclerosis). In the 1960s, at Glasgow University (UK), R. Cole, R.G. Edwards and J. Paul (1964 and 1966) isolated the first colony of ES (immortal) from rabbit blastocysts, and R. L. Gardner (1968) demonstrated the differentiation capacity of a single ES in the mouse. In the decade that followed, M. J. Evans (1972) isolated and characterized mouse ES, while R. A. Fleischmann and B. Mintz (1979) injected hematopoietic SCs into the placentas of immunosuppressed mice and thus cured a genetically determined anemia. In the 1980s, M. J. Evans and M. H. Kaufman (1981) isolated and established the first mouse ES line, and S. Fishel (1984) obtained the first human ES. The 1990s witnessed many advances; of particular note, A. M. Wobus (1991 and 1995) obtained heart cells from mouse ES, and E. Y. Snyder (1992) demonstrated that neural ES are transplantable and that they take root in mouse cerebellum. The first human (3 male and 2 female) and macaque monkey SC lines were prepared by J. A. Thompson (1998), while M. J. Shamblott (1998) obtained the first lines of human germ cells.

The legislation that allows the cultivation of human ES from blastocysts on the fourteenth day of development ordain the exclusive use of blastocysts that come

from in-vitro fertilisation clinics and that are therefore excess embryos that have not been transferred into the mother's uterus, but that, with the parents' consent, are used for research. Some countries, including the United Kingdom and South Korea, are debating whether to legalize the sale of oocytes by women who are willing to receive injections of drugs (gonadotropins) that stimulate superovulation, and thus enable oocytes to be collected for research purposes. Some bioethicists believe that this practice is unacceptable, and assert that, if legalized, it must be treated like organ donation and as such it must preclude any form of payment or future benefit from the associated research results.

At the beginning of the current century (2000), S. H. Lee produced dopaminergic neurons from mouse ES. In 2001, N. Lumelsky obtained insulin-secreting cells from mouse ES, and various Scandinavian groups confirmed that the transplantation of neural SSCs, derived from aborted fetuses, are effective in the treatment of Parkinson's disease. In 2003, K. Hubner, H. Scholer and M. Boiani produced germ cells (oocytes) from mouse ES. In Seoul, in 2004, W. S. Hwang received 242 oocytes from 16 donors and derived several human SC lines, but he altered the results and reported false technique effectiveness percentages. The media response was to present all of Hwang's work as fraudulent, although this claim is groundless. Still in 2004, T. Barbieri obtained millions of dopaminergic neurons from a single human ES. From this year onwards the growth in SC research has enter a *fast and furious* crescendo that is quite difficult to account for. Thus, we will review papers the references of which refer to the relevant details of SC biology. The most relevant result of these last few years has no doubt been the achievement of Shinya Yamanaka and his colleagues (see below) on the induction of pluripotency in fibroblasts by the ectopic expression of four stemness genes [14], something similar to what can be obtained by exposing somatic cell nuclei to the action of cytoplasts in a test tube [15, 16] or to the oocytes environment by nuclear transfer [17, 18]. Yamanaka's results are such a conceptual paradigmatic breakthrough that Professor Sir Ian Wilmut [19] changed the name of the journal *Cloning and stem cell* into *Cellular Reprogramming*. To explain the reasons behind this decision he wrote: "… it is appropriate … rename the Journal with this very important, exciting, and rapidly expanding aspect of biology. … A completely new area of research was established by Shinya Yamanaka and his colleagues when they demonstrated that, simply by introducing four transcription factors, it is possible to reprogram skin cells from mice so that they become equivalent to embryo stem cells." There is a wide consensus on the expectation that these milestone results greatly enhance Yamanaka's prospects in Stockholm for a Nobel prize.

In the last few years, methods for the derivation, isolation and culture of SCs, as well as for the genetic reprogramming of terminally differentiated somatic cells (reprogrammed to become embryonic or embryonic-like again), have improved. It is this latter type of research that has produced the most important results, by demonstrating that at least in some cases (cytoplasts and stem cell genes), embryonic-like SCs can be obtained without having to confront the ethical problems that in some countries arise from the legal protection assigned to the embryos. In fact, it has been demonstrated that terminally differentiated cells can be genetically reprogrammed

to produce ES. Indeed, it is possible to restore to a somatic cell nucleus, taken for example from a biopsy, the totipotent characteristics it possessed in the zygote. This is possible in egg cells which have had their nuclei removed (i.e. enucleated oocytes), thanks to a technique called *somatic cell nuclear transfer*. An extremely important turning point in SC research was the cloning of *Dolly* the sheep and *Cumulina* the mouse. I. Wilmut and K. Campbell's experiments using sheep [17] and subsequent experiments by R. Yanaghimachi on the mouse [18], have shown that when the nucleus of terminally differentiated somatic cells is transferred into the cytoplasm of an enucleated egg cell, it is able, despite its actual genetic program, to acquire a new genetic program and to begin and end embryo development with the birth of a new individual. The genetic program of a terminally differentiated cell nucleus is therefore not irreversibly fixed but can be reversed, thanks to *reprogramming factors*, to that of an embryonic cell nucleus. These two studies stimulated research on the capacity for genetic reprogramming of non-human egg cells, and culminated on 17 January 2008 in the decision by the British Human Fertilisation and Embryology Authority (HFEA) to grant licenses to researchers who want to create cytoplasmic hybrids between human cells and other animal cells. The HFEA thus approved the creation of cytoplasmic hybrids with the use of animal eggs to incubate somatic nuclei of human cells, the aim of which is to chemically reprogram the human somatic nuclei to express the characteristics of ES cells: the derived blastomeres must be broken up, and the SCs derived, within the first 14 days of cultivation. It should be pointed out that in the 18 years of its existence, the HFEA has approved only three research requests that involved the creation of embryos and the use of *nuclear transfer* for the derivation of ES. The completely arbitrary wording in media coverage of this framework has led to great confusion about what experiments are legal. Lay people tend to believe that in the United Kingdom it is possible for researchers to create human chimera embryo or human cytoplasmic hybrid embryo types as and when they please, i.e. without going through the HFEA or any other controlling bodies. Or, even worse, Mr. Average thinks that it is possible to derive fantastical biological entities from the various lexical combinations of the two embryonic types: cybrid chimeras! Biomedical research has a long history of mixing together the genetic material of different animal species, including that of man. Three different biological entities can be obtained by mixing the cells or cell components of different species together: cybrids (cytoplasmic hybrids), stembrids (stem cell hybrids) and chimera. These biological entities are completely different from each another, as are the techniques used to obtain them. Cybrids and stembrids are cytoplasmic hybrids produced by the respective insertion of the somatic cell nuclei of a species into enucleated eggs of another species (cybrids) and into ES cells (stembrids). Chimeras are produced by the co-mixture either of the embryonic animal cells of different species, or of different strains of the same species, during the pre-implantation stage of embryonic development. Nuclear transfer, a cell microsurgery technique, is used to produce cybrids and stembrids. Chimeras are produced by means of chemical substances and/or mechanical forces that disaggregate the early embryonic stages (pre-implantation) of different species or strains; the isolated embryonic cells (i.e. blastomeres) that are obtained are mixed together

in varying percentages (but usually 50/50) and then re-aggregated into a chimera. Cybrids and stembrids are produced in an attempt to derive ES cell lines; cybrids are also used in the cloning of animals in attempts to preserve species at risk of extinction. Far from being new, the fusion of human and animal cells is standard practice in biological research. Examples of its application in recent years include: human genome sequencing in the late 1970s and 1980s; diagnosis of human sperm quality on the basis of its use to fertilize hamster oocytes; the creation of transgenic animals, both for the production of molecules with pharmacological properties (e.g. growth hormones) in the milk, serum or urine of various animals (mouse, pig, sheep, goat, rabbit), and for the production of animal models for human diseases. The creation of cybrid embryos between differing animal species has been practiced for more than a century: the creation of human-bovine cybrids that were developed to the blastocyst stage, and the derivation, by a Chinese group, of human ES from a human-rabbit cybrid, are well known undertakings. Research that uses nuclear transfer between two different cells has a long history in biology. As far back as 1886, August Rauber used small syringes to attempt interspecific nuclear transfers (frog-turtle) in order to understand the role of the nucleus and the cytoplasm in heredity. It was during this period that the great figures of experimental embryology (Loeb, Delage, Spemann) tried to clarify Weisman's germ plasma theory. They tried, in other words, to understand how cellular differentiation occurs during embryonic development. More than 120 years of pertinent research have witnessed many nuclear transfer experiments, both intraspecific and interspecific: the most famous are the previously mentioned intraspecific experiments that culminated successfully in the development, though in very low percentages, of the reconstituted embryo and fetus that in turn led to the birth of *Dolly* the sheep and *Cumulina* the mouse. The interspecific experiments, between species of the same genus, have been used for the most part, and quite successfully, to save species at risk of extinction. The significance of the fact that full-term pregnancies and live births are registered only in species of the same genus, for example bovine, ovine, equine and feline intra-genus species should be pointed out. Therefore, embryos produced between cow (or pig) oocytes and Antarctic whale or rabbit nuclei do not reach the blastocyst stage. What is more, interspecific nuclear transfers have already been used to obtain ES cells. In 1999, Joseph Cibelli and collaborators (from Advanced Cell Technologies) claim to have created a cybrid by introducing a human lymphocyte nucleus into a bovine oocyte and to have derived a human ES line. This led to many other discoveries [20]: of note, in 2003, James Byrne and colleagues transferred the nuclei of various types of human somatic cells into amphibian oocytes, thus definitively demonstrating that the cytoplasm of Xenopus oocytes is able to genetically reprogram human somatic nuclei to express the OCT-4 gene, (a gene which indicates the reacquisition of SC potential). Other groups too, from America, Korea and China, have announced the production of human-bovine and human-rabbit cybrids. Together these data highlight the fact that cybrids develop, in very low percentages, to blastocysts (in the copresence of human and bovine or rabbit mitochondria), and that it is rare for these to reach implantation. Instead, they start to degenerate when the enzymatic coupling mechanism of reactions governed by the mitochondrial

genome has to synchronize itself with the reactions governed by the nuclear genome. The HFEA's stance denotes great responsibility, and guarantees that in the assessment of researcher's requests, the various ethical positions on human embryos that circumscribe the research world are given serious consideration and attention. In their decision of 17 January 2008, the HFEA acknowledged the fact that the requests by English researchers for permission to introduce the nucleus of a skin cell from patients with Alzheimer's or Parkinson's into bovine oocytes have great scientific value. These researchers intend to disaggregate the cybrids after four days of culture and to derive ES lines in order both to study their differentiation and to test drugs still in the experimental stage. More generally, the idea is to construct cell models of neurodegenerative diseases in such a way as to be able to *isolate* the diseases in test tubes. Cybrids and stembrids are created in the hope of being able to dissect the molecular composition of oocytes, those extraordinary, miniature, self-contained molecular biology laboratories. Such dissection could help to understand which factors activate the initial chemical reactions that reprogram somatic DNA into embryonic DNA. It is important to note that quality control protocol requires retrovirus monitoring for any SC line produced. Current evidence suggests, on the one hand, that cross-species cybrids do not have the potential to develop into embryos and fetuses that are endowed with organs and that are thus able to complete the development process. By *development process*, we mean that which results in the formation of a new individual with autonomous circulatory and nervous systems (the occurrence of which constitute two of the gradualist criteria optionally used to identify a new individual). On the other hand, research strongly suggests that it is possible to use human cybrids to produce ES cell lines without the involvement of human oocytes. This notion has prompted new research into identifying those factors in the egg cell cytoplasm that reprogram the nucleus of somatic cells to return to their embryonic state.

9.4 Stemness Genes

This new type of research is pursuing two distinct strategies, which for the sake of convenience are here respectively characterized as *environmental/cytoplastic* and *direct/transfective*. The former strategy cultures the somatic cells to be reprogrammed in cytoplasmic extracts (cytoplast is the term used to describe the matter that composes an enucleated cell. It therefore acts on the cell's *environment* (the culture material) and activates genetic expression mechanisms on the basis of factors that are *extrinsic* to the genome itself but that occur in the cytoplast. As the history of biology and medicine shows, this is a very important field of research: if these factors can be identified, it will be possible to produce them both naturally (from oocytes belonging to other species: rabbit, bovine, swine, amphibian) and artificially (biochemical synthesis). Just consider the production of pharmacological molecules (e.g. certain hormones) from matter that is no longer biological (corpses,

organic liquids such as urine), but that is actually biotechnological (bioreactors with microorganisms engineered by implantation of the synthesis genes from the required molecules). To get a wide view on the genes already identified as master stemness genes and the way they interact, the reader can benefit from several seminal papers: Boiani and Scholer [21], Li [22] and Hemberger et al. [23]. In particular, Figure 1 of Li's paper, which depicts the signaling interaction and core transcription regulation network that governs the pluripotency state, clearly demonstrates that Oct4, Sox2 and Nanog are master transcription factors for the maintenance of the undifferentiated state and self-renewal while Sox17 [24] is (one of) the ES key transcriptional regulator(s) of differentiation: when Sox17 is repressed pluripotency-associated transcription factors *Oct4*, *Nanog* and *Sox2* are active while its expression downregulates those three genes and activates genes controlling extraembryonic endoderm cytodifferentiation. In fact, our understanding of the gene's network controlling ES pluripotency is becoming clearer with respect to the vague idea we had until few years ago. What we have learned is that the same gene(s) can act simultaneously in contrasting directions, both promoting and repressing pluripotency and/or differentiation; this is achieved thanks to the action of ante- and retro-grade mechanisms active in a conceptual model of *cross-antagonisms in binary lineage decisions* (see below). In other words, there is a circular relationship between extrinsic signaling and epigenetic modifiers remodeling chromatin conformation which leads to transcriptional and post-transcriptional regulation of pluripotency factors. Among the players of this remarkable phenomenon of crosstalk regulating self-renewal are microRNAs (miRNAs) that control, by repressing the translation of selected mRNAs, self-renewal and differentiation [25]. Figure 1 of the paper by Gangaraju and Lin [26] highlights this, and Table 1 of the same paper lists the miRNAs involved in all cell types and lineage fates both in SC and ES cells. The crucial role played by miR-21 and miR-296 in promoting differentiation (i.e. repressing self-renewal) and of miR-22 and miR-290-296 cluster in promoting self-renewal (i.e. blocking differentiation) is quite clear. More than words, the information depicted in Figures 3 and 4 of Hemberger et al [23] will teach the reader of this crosstalk between genetic – epigenetic networks, molecular executors – pluripotency – gene expression / silencing – stemness.

9.5 Induction of Pluripotency

The proof that the identity of differentiated cells can be fully reversed comes from several lines of evidence beginning in the 1980s with Weintraub's group, which showed that the forced expression of MyoD in fibroblasts induces myotube formation (see [27] for a review). The author's team [16] used the environmental/cytoplastic strategy to reprogram fibroblasts into ES-like cells by *growing* them in cytoplasts of mouse oocytes. The other research strategy uses a *direct* route by genetic engineering techniques to insert stem cells directly into somatic cells and thus to acti-

vate *intrinsic factors* of the genome. Both Yamanaka's (Kyoto University) and Thomson's (Wisconsin University) teams successfully used [14] retroviral transfection to insert four genes responsible for stemness (Oct4, Sox2, c-myc,Klf4). At ≈ 0.001 - 0.1% efficiency and stemness yields are still very low for both these strategies. However, the path has been cleared, and over the next few years we will discover how to isolate reprogrammed ES cells (cytoplastic strategy) and how to *clean up* the retroviruses used (transfective strategy).

We already have proof that thanks to a true epigenetic virus-free transfection approach, that of inserting the same four factors into human cells using DNA transposons (i.e. *piggyBac*, from the cabbage looper moth), reprogramming factors can be inserted into differentiated cells to create *clean* iPS [28, 29]. In fact, DNA transposons can easily be completely removed from the genome thus avoiding the risk of insertional mutagenesis occurring with the use of retroviruses, lentiviruses, adenoviruses and plasmids carriers. However, all of the reprogramming techniques still had very low efficiencies, which may be due to several reasons as Shinya Yamanaka tried to explain [14].

These kinds of experiments have also allowed the conceptual derivation of the principle of *transcription factor cross-antagonisms in binary lineage decisions* developed by Thomas Graf's group [27] to explain cell transdetermination and transdifferentiation in development and experimental biology. A great merit of Graf's group is the additional effort to framework these concepts in the context of Waddington's epigenetic landscape models [27; refer to Figs. 1, 3, 5] as did Hemberger et al. (see above) reflecting on the epigenetic dynamics of SC biology [23]. Another great paper by Zhou and Melton [30] clearly explains the conversion of one cell into another, as did Wernig's group [31] showing the "direct conversion of fibroblasts to functional neurons in vitro by only three factors (Ascl1, Brn2 and Myt1l)". The piggyBac transposon system has been developed to avoid permanently altering the genome, e.g. non-integrating adenoviruses and transient plasmids. However, all these approaches involve introducing foreign DNA, while the ideal is to induce pluripotency for cellular reprogramming just using chemicals or biophysical stimuli. Sheng Ding (Scripps Research Institute, La Jolla, California) managed to do so [32] using a mix of purified proteins (from the four Yamanaka genes) and valproic acid (a histone deacetylase inhibitor). Along the route of the protein only technique, Shen Ding's team also contributed a detailed analysis [33] on selected chemical compounds that regulate cell fate: synthetic small molecules and natural products that bind to nuclear receptors, histone- and DNA-modifying enzymes and protein kinases and signaling molecules (see Fig. 2 and 3 in [33]). The great advantage of this promising chemical approach is that it is open to the development of therapeutic strategies by stimulating endogenous cells to regenerate in vivo (see the special insight on regenerative medicine [34]), thus likely targeting the patients' own cells. This means that the world of iPS (induced pluripotent stem cells) is a step closer to the clinic with personalized medicine.

9.6 Stem Cells in Regenerative Medicine

Over the last decade, stem-cell-based therapy for cellular diseases has literally transformed medicine (and will undoubtedly continue to do so), as traditional tissue repair techniques have been complemented by regenerative medicine. Diseases that already benefit of SC therapies include myeloproliferative disorders (bone marrow transplantation), certain types of blood cancer, severe burns (artificial skin) and cornea damages. New treatments, which are still at an experimental stage, but which we could reasonably expect to be ready in as little as five years, will target for heart attacks, Parkinson's disease and diabetes. To complete the experimental scenario, it is important to note that several SC applications are already being developed for regenerative medicine. The conceptual paradigm underlying this innovative research is the understanding that biological reagents, namely SCs, are now available, and that they are the means by which tissues can be regenerated. Some approaches to regenerative medicine (e.g. for defects in bone growth) use SCs from the patients themselves (autologous cells) and induce either circulating SCs or SCs from certain histological niches to move towards the area in need of repair. Other approaches use the current ability to produce large quantities of differentiated cells in vitro: SCs are cultured in solutions of varying composition containing molecules which can trigger differentiation into all the cell types that make up tissues (renal epithelium, lung, liver cells, cardiomyocytes, dopaminergic neurons, motor neurons, bone cells, etc.). In tissue engineering, these large quantities of differentiated cells can be used to obtain three-dimensional, transplantable constructs. The process uses biopolymer *skeletons* to produce the *organ* required. Research and experiments are testing this technique on animal models in the pursuit of ambitious goals. For example, nude mice (deprived of their immune system) are used to produce human germinal cells, spermatozoids and oocytes; with pliable biomaterials serving as a *skeleton* on which to grow transplant organs, SCs are triggered to differentiate into the cell types that will form the organ in question. To date, success in the cultivation of transplantable organs is limited to the bladder (Anthony Atala, Winston-Salem, North Carolina). Attempts to grow teeth and the heart are in the early stages of development. Research into organ cultivation is underway in several countries, and covers the whole spectrum of systems that are prone to organ and tissue diseases: the nervous system (spinal injuries and neurodegenerative disorders), the cardiovascular system (repair of necrotic area after heart failure, blood vessels), muscle and bone (muscles, articular cartilage, production of collagen type I for skeletal diseases, tendons, ligaments), endocrine (diabetes type I). It is worth recalling a few examples of the therapeutic use of SC, while the reader is referred to other sections of this volume for a detailed analysis. The recovery from adenosine deaminase deficiency (resulting in severe combined immunodeficiency, ADA – SCID) in young patients [35, 36] by stem cell gene therapy is a good example of the potential use of the powerful technique of genetic modification of somatic SC [37]. The ongoing successful trials today cover a wide range of diseases that already find therapeutic ameliora-

tion thanks to SC treatments, i.e. the necrotic heart, Parkinson's loss of dopaminergic neurons and diabetes I. The latter has seen a great advancement, from islet transplantation strategy to in vivo reprogramming of differentiated pancreatic exocrine cells in adults (mice) into ß-cells [38] thanks to the re-expression of three key developmental regulators (Neurog3, Pdx1 and Mafa).

The first methodic clinical trial treatment based on human ES has also been given the green light although the study is currently deadlocked (February 2010) by the Food and Drug Administration (FDA). Geron Corporation (Menlo Park, California) received approval to treat eleven patients (in a phase 1 multicenter trial) who had suffered a complete thoracic-level spinal cord injury with the "GRNOPC1 product". This is a suspension of human ES cell derived oligodendrocyte progenitor cells that already showed in preclinical studies an amazing capacity to remyelinate and to stimulate nerve growth. This is the first time that the FDA has testified the acceptance of all the procedures used to grow and characterize a SC population. Before this approval there were only anecdotal cases of treatment, the most popular being the study by Prof. Evan Y. Snyder, who implanted SC harvested from placentas or derived from a variety of tissue types into patients with multiple sclerosis, muscular dystrophy and several other neurological diseases. Interestingly enough, the meta-analysis of more than sixty preclinical studies carried out by Janowski et al. [39] on the employment of intravenous cell delivery for treatment of neurological disorders showed that the indisputable positive effects of the therapy on brain functioning are due to the molecular inhibition of several apoptosis pathways.

A good example of how fast and furious the advancement of knowledge has been is given by the discovery by Songtao Shi of SC in dental pulp ten years ago: in such a short time period we are already at the first translational medicine clinical trials for regenerative medicine with autologous treatments (for which a full FDA approval is not required). One of these studies was carried out by an Italian team [40] that used dental SC to reconstruct the bone surrounding the site from which the dental pulp was extracted. In addition, since these SC originate from the neural crest they have the potential to treat neurodegenerative diseases as shown by the successful neuronal growth detected after their grafting into the hippocampus of immunosuppressed mice [41].

Scientific development apart, the legal and ethical issues must undoubtedly be resolved before these experimental models can be applied clinically, whether to treat patients, or to relieve the transplant crisis, or to use SC to perform *personalized* medicine.

9.7 International Legal Framework: Stem Cell Biopolitics and Scientific Citizenship

SC technology challenges many of the principles of modern culture, such as freedom, progress, democracy and justice; it affects all aspects of our life and our

health. Internationally, we note the conspicuous absence of a politico-cultural stance such as might identify the points of contact between advances in scientific research and the autonomy of individuals over bio-existential choices. This absence is partly due to the fact that in recent years knowledge has progressed tumultuously. More generally, however, the relationship between citizens and the state on biopolitical issues has not been redefined, and the same may be said of the relationship between democracy and rights. The scientific community needs political decision-makers to develop valid policy guidelines upon which to base the management of the wide-ranging issues generated by the biotechnological revolution and by SC research. Of extreme importance and urgency is the governance of biotechnology research, a process to which all citizens should be able to contribute. To do so, citizens will have to expand their understanding of the intrinsic opportunities and limitations inherent in biotechnology, and particularly in SC biology and techniques. Ideally, each individual should be able to constantly re-delineate the boundaries of his relationship with the world, and thus to stay abreast of the changes wrought in boundaries, as well as in the deeper meaning of life and its forms, by our era's constant advances in knowledge: the biopolitics of the body and its transformations, birth, end-of-life decisions, biomedical experimentation and control of personal decisions, are all at issue. Only through the development of reflective attitudes will citizens avoid the *easy routes* in debate, e.g. the opinion that the research described here "will lead to technology taking control over humans" would be (horribly) *easy*. A perfect example of the impediment of the unreflective, *easy route* thinking is to be found in the story of cloning.

The story highlights other limits of knowledge-based Western societies: ideological preconceptions and prejudices. On an international scale, with more or less emphasis, political decision-makers tend to discuss the nature of the embryo without paying attention to what researchers or scientists have to say. Typically, there is a reluctance both to make scientific information readily available, and to allow citizens to express themselves freely on constraints and limitations, or even on those possible applications that are deemed lawful. The general public is manipulated with poor information and scaremongering about what the mad scientists would like to do. The result is a huge delay in possible therapeutic applications for Parkinson's, dystrophy and juvenile diabetes, to name but a few afflictions. The most dramatic example of this is how some countries fail to deal with cryopreserved embryos, when the simple application of a scientific method could help bring the complicated debate on bioethics to an end. There are three ways to deal with frozen embryos (adoption is excluded from the list because it is impractical and unrealistic: In Italy only two or three embryos a year are implanted into adoptive mothers):

1) leave them frozen indefinitely. This decision is synonymous with death, albeit slow;
2) defrost and discard them, thus accelerating their death;
3) use them for research into cell differentiation. This option also implies death, but by providing humanity with important scientific knowledge on how to use cells in reconstructive cell therapy, it would arguably be an attenuated form of death. And indeed *reconstruction* also implies a living embryo, albeit in an

extended sense, because the embryo's cells would be disseminated to other living individuals.

Looking beyond ideological, religious and ethical standpoints, it is clear that only the third option ensures the embryo's life. The decision should be based on *what to do* instead of considering *what they are*. It is important to stress once again how the application of a scientific method could help decide what to do with hundreds of thousands of frozen embryos, instead of inappropriately referring to ethical principles or to the concept of *person*. This concept does not belong to biology or to factual science, and is only valid in philosophy, law and theology: the *character* mask in Greco-Roman theatre, and the Holy Trinity and Christ as a person. Many religions consider animals and hurricanes to be people with souls, just like human beings. One suggestion might be to find an operational definition of ethics, the theory and practice of the conduct aimed at finding happiness through being good. Aristotle thought that happiness and good were virtues, whereas Kant thought they meant having the autonomy to behave according to universal laws. But it is not easy to determine the factual nature of being good. Indeed, it is clear that the alleged ethical consensus of humanity continues to be a beguiling hypothesis: man's exploitation of man, the just war and human history in its entirety, suggest that the hypothesis is probably false. Ethics are determined by religion and ideology. Religion entails adhesion to a view of the world in which life is constrained (relegated) to the notion of fundamental choice, to the extent that you would even give your life in support of this choice. Ideology is a vision and assessment of the world marked by social transcendence (political ethics in Aristotle). Therefore, we cannot expect a Hindu, a Christian and a dialectical materialist to follow the same ethics. In today's complex world, only responsibility can assist decision-making, since the basic element of ethics is responsible conduct (conscious and voluntary), and therefore, making a choice. These embryos exist and demand a better fate than being kept frozen indefinitely or being discarded: now that they have been created, they petition us to play a part in the matter-energy process that we call life. Scientific method could clearly be of assistance if we were to re-collocate our own legitimate principles within an awareness of ongoing advances in scientific knowledge. Keeping abreast of such advances is an integral part of our culture; it requires patient dedication to acquire the conceptual instruments needed to assess technical applications.

Today, in the age of knowledge-based societies, those instruments are necessary, especially for society's leaders, such as political decision makers, magistrates and journalists. The *generation gap* that affects attitudes towards the technical reproducibility of certain phases of embryo development is once again paradigmatic, and it is hindering all SC research in Italy, Austria, Germany and Ireland. As already stated, this hindrance certainly derives from the refusal of a scientific approach to the problem. It is clear that differences of opinion about the embryo derives from disagreement on exactly when in the development process the cells become a new individual. An individual is composed of about one million billion cells and originates from a development process that is programmed in the first copy of the new individual's DNA, the zygote genome. This affirmation has been ascertained as fact: biological knowledge allows us to clearly establish that the beginning of the devel-

opment process coincides with the formation of the first *functional* copy of its genome. All forms of animal and plant reproduction, both natural (fertilization and parthenogenesis) and artificial (assisted reproduction and cloning), share this principle; the principle accordingly assumes a universal validity that shelters it from all doubt whatsoever, and scatters all other propositions as to the ontogenetic origin of a living being. In mammals, this originating moment does not coincide with the appearance of the unicellular embryo, the zygote. It varies on the basis of the species in question: in mice, it occurs when there are two cells, in humans, when there are four. Acknowledgement of this factual datum would allow advocates of all religious and ideological standpoints both to defend their principles and to realign their positions to a setting within the boundaries of current scientific knowledge. At least two positions collapse as fallacy (i.e. as lacking universality): the gradualist view (whereby the human individual begins to exist when the nervous system appears, around either day 14 of gestation, or days 6-7 in the case of uterine implantation); and the view that considers fertilization (the fusion of sperm and oocyte membranes) as the beginning of a human life. Indeed, not all living creatures form a nervous system, become implanted in the uterus, or exist through fertilization (e.g. babies born following ICSI treatment never passed through fertilization in the natural way, i.e. through the sperm and egg membranes fusion). The four-cell embryo occurs between the 40^{th} and 50^{th} hour of development in humans, so the doctor would have time to produce the number of embryos needed, to perform pre-implantation diagnoses, and to derive SCs, without having to enter into philosophical debates on the nature of existence, or play semantic games on the meaning of the word *embryo*.

Developments in life sciences raise wide-ranging collateral issues (social, legal, political, economic, religious and philosophic), and the breadth of these issues is central to any analysis of the transformation of Western societies. Of particular importance is the role of politics and the extent to which the political system: (a) recognizes alternative policies as valid within a democratic framework and (b) invests in projects that promote shared values. Such values are an invaluable asset in a multicultural society, and science can contribute to their development. For a society to be democratic and based on justice and equity, its citizens need to be scientifically literate and knowledgeable. Only citizens who have the conceptual tools to critically evaluate the new frontiers of scientific knowledge can ensure a democratic system, because they are able to influence society in an effective way with their own independent opinions.

The organization of new knowledge and the development of new models of representation are the basis for a new and necessary form of democracy, in which new knowledge is not seen as a threat but as an opportunity. Instead of being considered a source of inequality, knowledge should actually serve to promote the welfare of all humanity, as Francis Bacon proposed four hundred years ago. In order for this to happen, the biosciences need to develop new analytical tools, both for the assessment of science-driven revolution and for the delivery of clear explanations of the same to the general public, the lay-people; well-informed citizens guarantee both strong support for investing in scientific research, and the formation of independent

opinions that are reflected in democratic decisions about what should or should not be done. A good example of procedural correctness is to be found in the UK government's recently published "White Paper on Genetics in the NHS"; addressed to all citizens, it establishes democratic monitoring, and ensures respect for the elaboration of principles and ethical norms that permit multiple values.

The biological knowledge available to us today puts many of the principles of modern culture to the test: freedom (e.g. the opportunities that genetic databanks offer medical research or criminal investigation), progress (opportunities in finance and in medical diagnosis), and even democracy and distributive justice; it intervenes in all areas of individual and social life. Biology's manipulation of living organisms (think of the substantial opportunities offered by GM technologies) has effectively brought about a scientific revolution which has almost exclusively been considered in terms of technological progress. Accordingly, the impact of such manipulation on modern culture has not yet been sufficiently debated and assessed. This leads us to ask a philosophical and political question; should we refuse scientific progress (which would lead to a genetic class system, as any new advances in medicine, for instance, would only be available to the people who could afford them), or should we speed up the processes of social awareness and management of the new relationship between science and society, which the new biological revolution has instigated? The true nature of scientific endeavor is democracy: advancement of scientific knowledge can mitigate or eliminate the inequalities that have historically characterized the life of all human beings. It could enable a new conception of citizenship which goes beyond that of Jefferson's American Revolution, or of the French Revolution: in the era of globalization and scientific knowledge-based societies (knowledge has evidently become the real economic and social driving force), citizens will all have equal access to SC therapies, and any other medical care they need, regardless of where they come from or how wealthy they are.

Well-informed, healthy citizens can guarantee a democratic, fair society.

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Cancer Stem Cells

10

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10.1 Origin and Evolution of the Cancer Stem Cell Paradigm

Far from being a new concept, the belief that cancer might originate from stem cells dates back to the mid-19th century when Rudolf Virchow proposed that cancer arises from embryo-like cells, based on the histologic similarity between embryonic and cancer tissues. This hypothesis was later extended by Cohnheim and Durante, who postulated that adult tissues contain embryonic remnants that usually lie dormant, but can be activated to give rise to a tumor. This original view, formerly referred to as the *embryonic rest theory*, has been updated with the *cancer stem cell hypothesis*, according to which a vicious stem cell-like subpopulation generates a tumor through the deregulation of the self-renewal process. However, the proof-of-concept was provided more than a century later with the identification of leukemia-initiating stem cells in the peripheral blood of acute myeloid leukemia patients [1]. Ever since, the cancer stem cell theory has gained identity and different tissue-specific cancer stem cells (CSCs) have been isolated from both *big killers*, and rare cancers. These studies were based on a similar experimental approach, which combined fluorescence-activated cell sorting (FACS analysis) of primary tumor cells with antibodies against specific cell-surface markers and serial orthotropic transplantation into immunocompromised mice. Although different operational definitions of CSCs have been proposed, four properties are in general adopted to define them: (i) expression of a distinctive repertoire of cell surface markers for isolation and purification; (ii) formation of tumorspheres in suspension culture; (iii) tumorigenic capacity in immunocompromised mice, as opposed to all other cellular subsets; and (iv) generation of a heterogeneous cancer tissue closely resembling the

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original tumor. A further technique for isolation of CSCs relies on their ability to actively exclude the dye HOECHST 33342, which defines them as side population (SP).

10.2 Functional Genomics of CSCs

Given the complexity and heterogeneity of genetic derangements, cancer is canonically defined as a genetic disease. In the last ten years, the introduction of whole genome profiling technologies has expanded our knowledge of the genetic pathways associated with the development and progression of cancer and, more recently, microarray analysis of gene expression profiles has provided a way to improve diagnosis and risk stratification of cancer patients. Two prognostic signatures, for example, the MammaPrint® and the Oncotype DX®, have been validated in breast cancer and represent the basis for large ongoing clinical trials named MINDACT (Microarray In Node negative Disease may Avoid ChemoTherapy) and TAILORx (Trial Assigning Individualized Options for Treatment). Since the major implication of the cancer stem cell theory is that key pathways controlling normal stem cells are deregulated in their malignant counterpart, gene expression profiling of CSC-associated genes might provide a more exhaustive picture of deregulated stemness-associated genes. The identification and validation of CSC-based signatures might have different implications including a more accurate individual risk assessment, the identification of new druggable pathways and the discovery of more specific markers for CSC isolation and purification. In a proof-of-principle study, a global gene expression profiling led to the identification of an *invasiveness* gene signature (IGS), consisting of 186 differentially expressed genes in breast cancer stem cells compared with normal breast epithelium [2]. The IGS was significantly associated with overall and metastasis-free survival in a large cohort of breast cancer patients. Using a similar approach, genes involved in maintenance of stemness and tumor-stroma crosstalk, such as hedgehog signaling and transforming growth factor-β (TGF-β) pathways, have been found selectively deregulated in breast CSCs, while nontumorigenic cancer cells express differentiation-related genes. Notably, TGF-β is a well-known mediator of the epithelial-to-mesenchymal transition (EMT) program which, in turn, is associated with the acquisition of stemness traits. With regard to anticancer therapy, a stem-cell-resembling expression profile consisting of an 11-gene signature has emerged as a powerful predictor of a short interval to disease recurrence and death after therapy in distinct types of cancer patients [3]. Finally, microarray analysis of SP versus non-SP thyroid cancer cells revealed a distinctive pattern of expression of stem-cell-associated genes, thus suggesting a potential role of high-throughput genomic screening for CSC isolation [4].

10.3 Molecular Biology of CSCs

A normal stem cell is defined as a low-frequency cell type able to renew itself and to differentiate into one or more specialized cells. In general, self-renewal, extensive proliferation and capability to differentiate into multiple lineages are the hallmarks of stem cells, which make them unique. The self-renewal program, fluctuating from symmetric to asymmetric divisions, is the most important variable for stemness maintenance in both physiology and pathobiology. The symmetric division implies the generation of two daughter stem (or two more differentiated) cells, whereas during the asymmetric division the progeny consists in a cell retaining a stem cell phenotype and a more differentiated cell. Two major classes of normal stem cells are to be distinguished: embryonic stem cells (ESCs) and somatic stem cells (SSCs). The first are pluripotent progenitors derived from early embryos able to generate the three germ layers and then, under a strict differentiation pattern, all differentiated cell types. SSCs have a much more restricted potential, representing a dedicated pool of undifferentiated and slowly cycling cells located in various organs of the body. This cellular subset maintains tissue homeostasis by replacing senescent, dying or damaged cells through a high capability of both self-renewal and asymmetric division. Thus, SSCs play a critical role in sustaining the lifelong functionality of organs. In adults, the functional triad of SSCs is spatially and temporally regulated into specialized microenvironments, named niches, in which stem cells reside. These highly organized micro-architectonic entities, mainly constituted by mesenchymal cells, provide physical anchorage and govern the stem cell fate by controlling both their number and the replication kinetic. To date, the most advanced studies in stem cell niche research have been carried out in the hematopoietic system [5]. Within the bone marrow two different niches have been described and characterized. The osteoblastic niche controls hematopoietic stem-cell cycling and promotes quiescence, whereas the vascular niche mainly regulates proliferation and egress to the circulation. The shuttling of hematopoietic precursor cells between the two niches is probably dependent on the oxygen level. Under hypoxic condition, hematopoietic stem cells move from the osteoblastic to the vascular niche, thus providing the necessary supply of mature cells in the peripheral blood stream. The opposite path takes place with the re-establishment of a normoxic state. Since CSCs probably share various properties with their normal counterpart, it is currently believed that these tumor-initiating cells act by hijacking physiologic stem cell signalings and require a niche-like supportive microenvironment. From a functional point of view, this notion implies that developmental regulatory molecules and/or pathways orchestrating the self-renewal program in adult tissue stem cell compartments, such as wingless-type-β-catenin, notch-jagged, hedgehog-patched-smoothened and bone morphogenetic proteins, represent the major controllers of CSCs. For example, the hedgehog signaling pathway has been found to be reactivated in some skin and central nervous system cancers, like basal-cell tumor [6] and medulloblastoma [7], in consequence of loss- or gain-of-function mutations in the

negative (patched homologue 1, PTCH1) or positive (smoothened homologue, SMO) regulator of the pathway, respectively. These mutations result in constitutive activation of the hedgehog signaling, leading to unrestrained proliferation of early progenitor cells of the skin and cerebellum. Similarly, activating mutations in the Wnt-β-catenin axis result in colonic polyps in consequence of hyperproliferation of intestinal crypt progenitor cells [8]. Considering the similarities between CSCs and their normal counterpart, it is also likely that CSCs rely on a tumor-promoting microenvironment that maintains CSCs able to self-renew, differentiate and proliferate. A paradigmatic example has been observed in AML, in which leukemic stem cells can displace and supplant normal hematopoietic precursor cells from the bone marrow niches.

10.4 CSCs and Carcinogenesis

Carcinogenesis is defined as a multi-step process in which both exogenous and endogenous factors concur in determining the accumulation of DNA mutations. The progressive acquisition of random, non-lethal transforming mutations finally turn a normal cell into a malignant cell. Historically, the *stochastic* or *clonal evolution model* of cancer has been widely accepted for decades. According to this model, different dominant mutant clones able to self-renew themselves acquire a survival advantage over other populations cohabitating within the tumor mass. Since different malignant cells maintain and propagate the tumor, the heterogeneity seen in cancer mirrors the natural competition between various dominant clones for vital resources, according to Darwinian principles. Even if the accumulation of genetic lesions underpins this model, also epigenetic changes and microenvironmental factors cooperate to finally generate a malignant phenotype. On the other hand, the concept that a CSC represents the seed of tumors implies that a stem cell is the initial target of the oncogenic process and, although CSCs constitute a minority of the cells within a tumor, this subpopulation is critical for tumor propagation. In general, the higher proliferative potential and the longer life-span of stem cells, compared with their differentiated progeny, make these cells more likely to undergo and accumulate genetic alterations. In recent years, the identification of these tumor-initiating cells led to the paradigm of cancer as a heterogeneous, organ-like micro-ecosystems in which the CSC is positioned at the apex of the tissue hierarchy, thus closely resembling the functional architecture of normal tissues. As a result, the histologic, genetic and molecular heterogeneity of tumors, as well as the interindividual variability in the natural course of neoplastic diseases, could be explained with the intrinsic plasticity of a CSC which retains, albeit aberrantly, many features of its normal counterpart. Although this hierarchical model with a small pool of stem cell-like tumor-initiating cells at the top of the pyramid has largely replaced the *clonal evolution model*, both models are not mutually exclusive, but probably coexist, since CSCs themselves may undergo clonal evolution.

10.5 CSCs and Tumor Recurrence

The widespread adoption of screening programs and the refinement of radiologic techniques have led to a dramatic increase in patients diagnosed with early-stage disease, thus suitable for treatment with a curative intent. If radical surgery is the mainstay of treatment for these patients, perioperative radiotherapy and/or systemic therapy are often administered in order to decrease the likelihood of loco-regional and distant recurrence, respectively. Despite these efforts, the global mortality rate for many tumors remains quite stable. This is, at least in part, attributable to distant recurrence. The pattern and the time of recurrence of many tumors, often in the order of years after the primary treatment, imposes three major biologic considerations: (i) the acquisition of malignant traits that enable cancer cells to colonize distant sites is an early event, as corroborated by the detection of circulating tumor cells and/or disseminated tumor cells in the bone marrow of early-stage cancer patients (minimal residual disease); (ii) early-disseminated cancer cells can remain in a quiescent status for years, a condition known as *tumor dormancy*, which explains tumor relapse after a long period of remission; (iii) residual cancer cells, or at least a fraction, are intrinsically resistant to radiotherapy- and chemotherapy-induced cell death, thus impairing (neo)adjuvant therapy. There are many biologic clues supporting CSCs as the ideal candidate for explaining the failure of this integrated therapeutic approach. First, increasing evidence is connecting CSC biology to the EMT, which is thought to be one of the major determinants in cancer invasion and metastasis. The EMT is a genetic program consisting in a drastic cytoskeletal rearrangement through which cancer cells at the leading invasive edge acquire a high motile, mesenchymal-like phenotype. It has been demonstrated that the induction of EMT results in the acquisition of stem-cell like properties, such as an increased ability to form spheres in suspension culture [9]. Moreover, the ability of normal stem cells, and probably of CSC, to remain in a quiescent state within a protective niche provides the conceptual framework of tumor dormancy. In light of this, the observation that breast cancer-derived bone marrow micrometastases contain a higher percentage of cancer cells expressing *stemness* markers compared with the primary tumor site further enforces this assumption [10]. Other properties of CSCs, such as the constitutive activation of the DNA repair checkpoint and a strong expression of radical scavenger genes, can be linked to local and locoregional recurrence following adjuvant or salvage radiotherapy. Moreover, the failure of systemic adjuvant chemotherapy and the progressive acquisition of a chemorefractory phenotype suggest that adaptive mechanisms are brought into play by CSCs in order to circumvent chemotherapy-induced cytotoxicity. Schematically, it is possible to distinguish at least four independent mechanisms of chemoresistance [11]: (i) an increased expression of multidrug-resistance proteins; (ii) an efficient system of DNA repair; (iii) strategies to avoid apoptosis; and (iv) capability to remain in a quiescent state. CSCs express high level of drug pumps such as adenosine triphosphate (ATP)-binding cassette transporters (ABC transporters), which provide the rationale

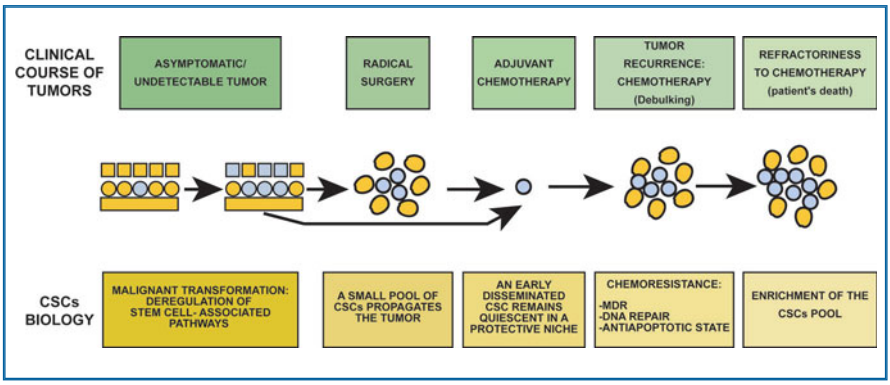

Fig. 10.1 The clinical course of tumors which relapse at distant sites years after surgical treatment and which progressively become unresponsive to chemotherapy can be explain by the biological properties of CSCs

for the HOECHST 33342 efflux assay. This phylogenetically conserved system for drug efflux extrudes a broad range of amphiphilic compounds, also including a wide range of chemotherapeutic agents of natural origin such as mitotic-spindle poisons. This phenomenon is known as multi-drug resistance (MDR). An enhanced capability to repair DNA damage can confer resistance to alkylating agents. It is well known that the overexpression of proteins playing a critical role in the nucleotide excision repair (NER) pathway, such as ERCC1 (enzyme repair cross-complementation group 1), makes cancer cells more proficient in removing platinum-DNA adducts. Next, harmful damaged cells are physiologically removed by programmed cell death, or apoptosis. Conversely, the imbalance in the apoptotic machinery is associated with chemoresistance. CSCs express high levels of anti-apoptotic proteins such as BCL-2, BCL-XL and IAP family members, which confer a proclivity towards an anti-apoptotic state. Moreover, quiescent or dormant CSCs might be intrinsically resistant to chemotherapy since such agents are exclusively effective against active cycling cells. Finally, an enrichment of the CSC pool has been documented following exposure to different chemotherapeutic agents. This latter phenomenon has a dual, biologic and clinical, implication since, on the one hand, it represents a crossroad between the *clonal evolution model* and the *hierarchical model* and, on the other hand, it might explain the progressive chemorefractory state that finally leads to patient death. Figure 10.1 shows the natural course of tumors according to the CSCs theory.

10.6 CSC and Metastases

Once metastasized, a tumor becomes incurable and palliative systemic therapy is the only therapeutic option which provides some benefits in terms of delay in tumor

progression and relief from tumor-related symptoms. For many years, a passive metastatization model based on the escape of cancer cells from the primary tumor, the subsequent access to systemic circulation and the final entrapment in the microvascular bed (*mechanical trapping* theory) has been accepted. However, this model does not explain the organ-specific metastatic pattern of many cancers, as well as the unusual involvement of some organs such as heart or kidney. Although the site-specific spreading of tumors was guessed since 1889 by Stephen Paget, it was only during the 21st century that the *seed and soil theory* has taken more concrete shape. According to a modern view, the metastatic cascade occurs in a stepwise, nonrandom fashion and requires a series of coordinate events ranging from the generation of new blood vessels to adaptation to growth in a foreign soil. Metastasis formation is far from being an efficient process since only a limited number of cells, once having left the primary tumor, can successfully complete all steps required to re-grow in a distant site. In such a scenario, therefore, the discovery of CSCs has added a further level of complexity about mechanisms orchestrating cancer metastasis. Based on the assumption that a plastic CSC is the tumor-initiating cell, it is reasonable that a solitary CSC can initiate a metastatic lesion and then thrive in a tumor-hostile microenvironment. To reach a distant site, however, cancer cells have to accomplish early phases of the metastatic cascade, which enable them to enter the systemic circulation and, afterwards, to migrate toward the future site of metastasis. The generation of new blood vessels, a process known as neoangiogenesis, represents a rate-limiting process in growth and survival of both primary tumor and metastasis. In fact, a tumor cannot grow beyond 2 mm in the absence of an adequate vascular supply. Through neoangiogenesis cancer cells not only gain access to oxygen and nutrients, but also can enter the systemic blood circulation. This complex and dynamic process is mainly sustained by vascular endothelial growth factor (VEGF) family members and their cognate receptors (VEGFR family members), which trigger a multitude of proangiogenic activities. Evidence on how CSCs influence neoangiogenesis comes from studies on high-grade primary nervous system tumors. Under both normoxic and hypoxic conditions, stem-cell-like glioma cells produce higher levels of VEGF than the non-CSC population, thus suggesting a potential role of CSCs in the angiogenic switch [12]. It is, however, likely that within a heterogeneous tumor mass some clones do not rely on angiogenic programs, but adopt alternative strategies to recreate a vascular bed including vasculogenesis (de novo generation of vessels from pluripotent stem cells) or vascular mimicry (tumor cells mimicking endothelial cell functions). It has been demonstrated, for example, that under specific tissue conditions ovarian CSCs differentiate into endothelial-like cells [13]. Thus, CSCs actively contribute to tumor vascularization by serving, ultimately, as vascular progenitors. Although it is not clear to what extent CSCs are dependent on a protective microenvironment (the vascular niche), brain tumor-initiating cells localize near the capillaries [14]. In these areas endothelial cells probably maintain CSCs in an undifferentiated state via the regulation of the self-renewal program. Once within the blood stream, cancer cells migrate in a nonrandom manner, probably driven by a complex network of chemoattractant cytokines that physiologically control leukocyte trafficking and stem cell movements within the

bone marrow niches. Ample evidence suggests that the chemokine receptor CXCR4 dictates the migratory itinerary of cancer cells once engaged by its exclusive ligand CXCL12. This migratory response probably exploits a chemokine gradient since CXCL12 is constitutively produced in the most common sites of cancer metastasis such as bone, lymph nodes, liver and lung. Notably, CXCR4 has been found to be expressed by several cancer cells and has been proposed as CSCs marker [15]. It is, therefore, likely that CSCs adopt CXCR4 to selectively migrate toward, and then colonize, distant organs expressing a high amount of CXCL12. The role of EMT in cancer metastasis is discussed in Section 10.5.

10.7 In Vivo Imaging of CSCs

In clinical practice the extension of a tumor and the response following systemic therapy are assessed by morphologic imaging technologies, such as computed tomography (CT) and magnetic resonance imaging (MRI), or metabolic techniques like positron emission tomography (PET). Given that CSCs represent a rare subpopulation within a tumor, the CSC model raises the question of a single-cell or single compartment resolution. Albeit with a certain degree of variability, in fact, CSCs are in the order of 0.1% in primary tumors and commercial cell lines. However, their biologic relevance imposes the development of new imaging techniques and/or the adaption of preexisting high-resolution radiologic technologies to detect and monitor them. Even though ex vivo imaging of cancer cells at a single-cell level was made possible a number of years ago, in vivo imaging of CSCs is a more complex challenge. In particular, the critical point is the choice of the optical signal. Although bioluminescence is widely used to define tumor growth and metastasis in vivo, bioluminescence imaging has intrinsic limitations. This technique is far from a single-cell resolution since it needs approximately 2,500 luciferase-expressing cells for a proper visualization [16]. Moreover, both the need to inject animals with the luciferin and the biodistribution of the substrate should be considered as potential limiting factors. Fluorescent signals represent the gold standard for tracking a poorly represented population of cells [17]. The detection of fluorescence offers three major advantages: high intensity and stability of the signal, high sensitivity of the optical imaging devices for fluorescence readout and the possibility to concomitantly employ two or more fluorophores, thus allowing the concomitantly visualization of different populations within a tumor mass. Furthermore, the surgical creation of a skin flap allows internal organs to be imaged by avoiding skin-related light scatter. Beside fluorescent and bioluminescent signals, different probes are available to detect microenvironmental changes, such as neoangiogenesis or extracellular matrix degradation, thus providing an overview of the whole tumor-stroma compartment. However, given the long experience gained with the development of MRI and PET, these technologies have more possibility to be adopted in the clinic with the specific aim of monitoring the CSCs pool. This has become a more concrete option

through the evolution of contrast agents for MRI, which have given the opportunity to image at a cellular resolution [18].

10.8 CSCs and Colon Cancer

Colorectal cancer (CRC) is a major public health problem, representing the second most prevalent malignant disease in industrialized countries. Despite radical surgery, approximately 40-50% of patients who undergo curative resection will experience distant recurrence. In addition, 20-25% of patients present with metastatic disease at the time of diagnosis. Although third-generation regimens incorporating targeted agents into the chemotherapy backbone are able to produce tumor response in nearly half of metastatic patients, the progressive development of a chemorefractory disease ultimately leads to patient death. In order to provide a more comprehensive picture on how colon CSCs (CCSCs) impact on the course of the disease, a brief examination of the functional organization of colonic stem cells in the context of their microenvironment is required. The normal histology of colonic epithelium consists of tube-shaped units, called crypts of Lieberkühn. Stem cells are located at the bottom of the crypt and through self-renewal generate a transit-amplifying population that, during migration up the crypt, terminally differentiate into colonocytes, goblet cells or enteroendocrine cells. Moving toward CRC pathobiology, the pioneer study of colorectal carcinogenesis came from Vogelstein who two decades ago described the adenoma-carcinoma sequence. This model was based on sequential mutations occurring in oncogenes and tumor suppressor genes that, through a progressive acquisition of neoplastic features, finally turn a normal epithelium into an invasive tumor. Within this framework it can therefore be assumed that it is a tissue stem cell located in the depths of the intestinal crypt that undergoes neoplastic transformation and progressively acquires pro-metastatic properties. This is consistent with histologic evidence showing multiple differentiated cell types in CRC samples. CCSCs have been identified by the expression of the cell-surface marker CD133 [19], also known as prominin-1. Although this molecule seems to act as organizer of plasma membrane topology, its functional role is still debated. However, according to the operational definition of CSCs, only the CD133+ population (accounting for approximately 2.5% of all tumor cells) is able to recapitulate the parental tumor in immunocompromised mice, thus giving rise a tumor with a ratio of CD133+ and CD133- cells similar to the tumor of origin. A further cell surface marker repertoire, consisting of $CD44^{+}/EpCAM^{high}$, identified a cellular population that displays the common features of CSCs [20]. More recently, beside these two protocols, aldehyde dehydrogenase 1 (ALDH1) has emerged as a promising CCSC marker [21]. Since all the above-mentioned protocols allow a cellular subpopulation with features of CSCs to be isolated, it is reasonable to assume that a combination of these markers could improve the isolation rate or, alternatively, could help distinguish CSCs with distinct biologic behavior. However, whether a

multiple-marker approach translates into a more efficient isolation of CCSCs has yet to be defined.

In order to shed light on CRC metastases, a *migrating CSC model* has been proposed. Consistent with this theory, it is currently believed that a mobilized CSC arises from a stationary CSC via the persistent activation of self-renewal-associated pathways and/or of the EMT program. This idea is corroborated by the fact that metastases from many tumors recapitulate the polarized pattern seen in the primary tumor, in which differentiated and undifferentiated cells reside in distinct areas of the tumor. Finally, while CSCs have been defined as the *dark seed* of cancer, this is mainly because of they are insensitive to treatment. Cytotoxic agents, in fact, are active against actively proliferating cells, representing the bulk of tumor mass, without affecting slowly-cycling (or quiescent) CSCs. As a result, chemotherapy kills differentiated, non-tumorigenic cells, leaving behind the CSC pool, or even its enrichment.

10.9 Breast Cancer and CSCs

Despite advances in early diagnosis and treatment, breast cancer (BC) remains a significant public health concern, with more than a million new cases diagnosed annually. To date, prognosis and treatment of early-stage BC are largely dependent on well-known clinical and pathologic features including age, menopausal status, tumor size, grade, nodal involvement, hormonal receptors expression and HER-2 status. Despite major advances in the characterization of the biologic aggressiveness of the disease, survival data reveal a wide variability in BC course within each prediction category, thus indicating that as yet uncharacterized molecular differences are responsible for such heterogeneity. In recent years, whole genome profiling technologies offered a working model for a BC molecular taxonomy. The original classification proposed by Perou, named *the molecular portrait of BC*, led to the identification of five subgroups (luminal A, luminal B, basal-like, HER2, and normal breast-like), according to the resemblance between the genetic profiles of normal and neoplastic breast epithelial and mioepithelial cells. Each group mainly differs by the presence or the absence of the estrogen receptor (ER), the progesterone receptor (PgR) and the amplification/overexpression of the protoncogene Her2-neu. Moreover, the presence of germline BRCA1 mutations, which predispose women to a lifetime risk of developing BC of up to 80%, make the picture even more complex since such mutations identify a further clinical and molecular entity. BRCA1-mutant BC are characterized by early onset, extremely aggressive biologic behavior and, probably, a different spectrum of sensitivity to chemotherapeutic agents, compared with all other subtypes. Taking into account this heterogeneity, the prevailing concept is that a different *cell of origin* is the seed of distinct subtypes. In the mammary gland, tissue stem cells able to undergo multilineage differentiation give rise to all three lineages (ductal epithelial cells, alveolar epithelial cells and myoepithelial cells) via lineage-restricted intermediates. This normal epithelial hierarchy

might explain, although in a merely correlative manner, the molecular and clinical heterogeneity of BC. Breast CSCs (BCSCs) have been characterized by the expression of cell-surface markers $CD44^{+}/CD24^{low}/lin^{-}$, comprising 1% to 10% of the total population [22]. Consistent with the hallmarks of CSCs, this subpopulation recapitulated the phenotypic heterogeneity of the parental tumor when injected into NOD/SCID mice. BCSCs can be also isolated by the expression of ALDH1. Considering a partial overlap between the $CD44^{+}/CD24^{low}/lin^{-}$ and the ALDH1-positive population, it has been found that as few as 20 cells $CD44^{+}/CD24^{low}/lin^{-}$ /ALDH1-positive are able to form a tumor [23]. If uncertainty exists about tumor-enhancing properties of other CSCs markers, CD44 is known to be involved in cell-matrix adhesion by binding hyaluronic acid, whilst CD24 is a negative regulator of CXCR4. With regard to ALDH1, it is interesting to note that this enzyme is able to metabolize chemotherapeutic agents, such as cyclophosphamide. Given the general principles of CSCs, it is reasonable to assume that the elucidation of stemness-associated pathways underlying breast carcinogenesis is the keystone to killing them. The Her2-neu, for example, is overexpressed in approximately 20-25% of human BC. If, on the one hand, Her-2 positive BC is associated with an aggressive clinical course, the development of Her-2 inhibitors such as trastuzumab and lapatinib have significantly improved clinical outcome. Since a direct relationship between Her-2 and ALDH1 has been documented, it is likely that Her2 plays a role in breast carcinogenesis and, at the same time, that anti-Her-2 therapy may be effective against BCSCs. In line with this, neoadjuvant anti-Her-2 therapy results in reduction of the BCSCs pool and an increase in the pathologic complete response rate [24]. Conversely, neoadjuvant chemotherapy-only regimens, which often achieve dramatic tumor debulking, result in an increased proportion of $CD44^{+}/CD24^{low}/lin^{-}$ cells, as analogously reported for other tumors. Bearing in mind that BCSCs share the general properties conferring chemoresistance treated in Section 10.5, it is worth noting that the multi-drug resistant protein ABCG2, also known as breast cancer-resistant protein, was initially described in BC. Beside the Her-2, other pathways involved in mammary organogenesis, such as Notch and Hedgehog, are thought to be the major determinants of BCSCs behavior and specific inhibitors (i.e. gamma secretase inhibitors blocking Notch signaling) are currently undergoing clinical trials. Beyond a therapeutic approach, the evaluation of the BCSCs pool could provide prognostic information. For example, the increased expression of ALDH1 seems to identify a subset of patients with inflammatory breast carcinoma with an increased risk of recurrence. The prognostic value of the 186-gene signatures is discussed in Section 10.2.

10.10 CSCs and Ovarian Cancer

Ovarian cancer (OC) is the most lethal malignancy of the female reproductive system. The high mortality rate of OC is due to the inability to detect the disease at

early stages, the high rate of disease recurrence and the development of a chemorefractory form. More than 90% of OC arise from the surface epithelium in consequence of ovulation-associated wound repair which, in turn, leads to abnormal expansion of the stem cell compartment. Epithelial ovarian cancers (EOC) is the most common type and includes various histologic subtypes mainly resembling the epithelial component of the lower genital tract. Each of these histotypes is characterized by a different genetic background. Thus, the existence of ovarian cancer stem cells (OCSCs) capable of unlimited self-renewal and multilineage differentiation may account for the histologic heterogeneity of OC. The first evidence connecting OCSCs to the aggressiveness of OC arose from the isolation of a single tumorigenic clone among a variegate population harvested from a patient suffering from ascitic effusion [25]. This clone was able to grow in an anchorage-independent manner (tumorspheres) and to establish, in animal model, a tumor having the histopathologic architecture of the human disease. Furthermore, the isolation of putative mouse ovarian CSC capitalized on the observation that stem cells defend themselves through MDR pumps, able to extrude harmful chemicals. As a result, the HOECHST 33342 efflux assay made possible the isolation of a SP representative of putative OC-initiating cells [26]. More recently, human OCSCs have been isolated by the expression of the cell-surface marker CD133 [27]. This CD133+ population possessed the operational criteria to define CSCs.

10.11 CSCs and Lung Cancer

Lung cancer is the leading cause of cancer-related mortality worldwide in both men and women. Approximately two thirds of patients present with advanced-stage disease, and a considerable percentage of patients with resected early-stage disease will experience distant recurrence. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases of lung cancer, while the even more aggressive small cell lung cancer (SCLC) makes up the remaining 15-20%. Regarding NSCLC, over the past two decades an *epidemiologic shift* has been observed and, to date, lung adenocarcinoma (LAC) has replaced squamous cell carcinoma (SCC) as the most common subtype of NSCLC. It is worth noting that while SCC is exclusively related to tobacco smoking, LAC and its variants (such as bronchiole-alveolar carcinoma) represent the most common histologic type in never and former smokers. Given the different etiology of LAC and SCC, as well as their differential molecular portrait and a partially different spectrum of sensitivity to some antineoplastic agents, the *dual face* of NSCLC has become evident. Thus, to date, LAC and SCC are considered two different clinical entities. As for other solid tumors, one of the most promising approaches to improving long-term outcome of patients is to define the source which maintains and propagates the tumor. According to the CSCs theory, in fact, such cells might exhibit vulnerability to targeted agents blocking stemness-associated pathways. The conceptual framework for targeting lung cancer stem

cells (LCSCs) is an exhaustive knowledge of the hierarchical organization of the normal airway system. Although in this context normal stem cells have not been identified yet, the topographic and functional organization of the respiratory tree support the existence of regional precursors such as bronchoalveolar progenitor cells, basal/mucous secretory bronchiolar progenitor cells and neuroendocrine progenitors, which maintain tissue homeostasis. Consistent with the CSCs model, tumorigenic hits occurring either in unidentified stem cells or in a more restricted precursor cells trigger the region-specific neoplastic transformation, thus providing a plausible explanation for the clinical heterogeneity of lung cancer. However, due to the lack of a representative repertoire of markers for the normal stem cell compartments, the identification of lung cancer tumor-initiating cells is far from being widely reproducible. Initially, a side-population identified by the Hoechst 33342 efflux displayed more tumorigenic proclivity in vivo and higher resistance to chemotherapy compared with the non-SP cells [28]. In spite of this, further attempts to identify phenotypic markers for this population have been disappointing. Only recently a rare CD133+ population isolated from both NSCLC and SCLC has been found to generate long-term tumorspheres in vitro and capacity to recapitulate tumor heterogeneity in vivo [29]. Probably, however, additional markers are needed for dissecting lung cancer heterogeneity. If phenotypic characterization of LCSCs is still challenging, understanding the functional forces driving them is even more complex. Since preclinical evidence suggests that both Notch and Hedgehog pathways play a role in determining the fate of LCSCs, the most promising mechanism of disease-based therapy is oriented to disarm CSCs by pharmacologically blocking CSC pathways.

10.12 CSCs and Pancreatic Adenocarcinoma

Pancreatic cancer is a highly lethal disease with an annual death rate almost similar to the annual incidence. The late diagnosis and chemo-radioresistance, combined with the intrinsic aggressiveness of the tumor all contribute to the dismal prognosis of pancreatic cancer patients. The median survival for patients undergone pathologically margin-negative resection is approximately 2 years with a 5-year survival in the range of 15%-20%. In the metastatic setting standard of care gemcitabine obtains a very modest benefit. Although debated, pancreatic acinar or centroacinar cells are the most suspected as the origin of pancreatic adenocarcinoma. Pancreatic CSCs (PCSCs) are defined by the expression of the cell surface markers CD44+CD24+/epithelial-specific antigen+(ESA+), accounting for approximately 0.2-0.8% of all pancreatic cancer cells [30]. According to the general criteria for defining CSCs, these cells are highly tumorigenic and possess the ability to both self-renew and produce differentiated progeny, thus mirroring the heterogeneity of the primary tumor. However, in human pancreatic ductal adenocarcinoma samples also a subpopulation of CD133+ expressing cells seems to embrace all the proper-

ties that define CSCs, with only modest overlap with the CD44+CD24+ESA+ phenotype. Among the CD133+ population, cells at the invading front coexpress CD133+ and CXCR4. Notably, whilst both CD133+CXCR4+ and CD133+CXCR4- cells are tumorigenic, only the CD133+CXCR4+ fraction displays metastatic proclivity. Taken together, these observations raise the question on whether different PCSCs populations, endowed with differential biologic aggressiveness, coexist within pancreatic adenocarcinoma. As expected, PCSC are resistant to conventional chemotherapeutic agents. Their treatment in vitro with therapeutic doses of gemcitabine results in morphologic and biochemical changes attributable to the activation of the EMT program, as well as in an increased expression of PCSCs markers [31]. Moreover, an enrichment of the CSCs pool following chemotherapy or exposure to ionizing radiation has been documented. Similar to other CSCs, the activation of developmental signaling pathways involved in the control of the self-renewal program are the major determinants of PCSCs fate. In line with this, the activation of the hedgehog pathway has been correlated with the development of cancer precursor lesions, while SMO inhibition by cyclopamine results in the arrest of cancer growth [32]. In conclusion, evidence is growing to support an important role for CSCs in pancreatic adenocarcinoma and novel targeted therapies blocking CSCs pathways are needed to really improve patient outcomes.

10.13 CSCs and Prostate Cancer

Prostate cancer (PC) is the most commonly diagnosed male cancer in Western countries. The increase in incidence is largely due to the combination of an aging male population and the use of screening tests based on the measurement of serum levels for prostate-specific antigen. PC can be efficiently treated by surgery if localized, but once metastasized it becomes fatal. Although PC initially responds to androgen deprivation therapy, the development of a castration-resistant disease is almost inevitable and the time to death is approximately 18 months, despite the improvement achieved with taxotere-containing regimens. The conventional model of PC implies that androgen receptor-(AR) expressing cells gain ability to spread and then re-grow in a distant site. This theory arose from the observation that the bulk population of tumor cells co-expresses AR and luminal cell-specific markers. In recent years, however, both the elucidation of the functional architecture of the normal prostate and the discovery of prostate tumor-initiating cells have radically changed this view. The prostate epithelium is a two-layered structure consisting of terminally differentiated luminal cells (the exocrine compartment) which rest upon an undifferentiated layer of basal cells. While luminal cells, representing the major cell type, express AR and are dependent on androgen stimulation for their survival, the basal compartment does not express AR and is independent of androgens. In their midst, a transit-amplifying population probably mirrors a continuum in the differentiation program. Because the basal stem cell is a long-lived cell, thus having more oppor-

tunity to acquire transforming mutations, it was not surprising that PC stem cells (PCSCs) have been identified through the same antigenic profile characterizing their normal counterpart ($CD44^{+}/\alpha_2\beta_1^{hi}/CD133^{+}$) [33]. These cells are likewise AR^{-} and express several *stemness* genes, including OCT3/4, BMI1, β-CATENIN and SMO. Such preclinical findings could provide a ready explanation for the minimal residual disease following androgen deprivation therapy. Although the hormonal-resistant disease has been largely associated, in fact, with AR amplification, activating mutations in the AR gene and/or alteration in the AR corepressor-coactivator activity [34], the intrinsic insensitivity of PCSCs to the androgen milieu might drastically change the future course of hormonal manipulations. By combining the notion that PCSCs do not rely on androgens with the observation that CSCs retain the protective mechanisms of normal stem cells (expression of ABC transporters, ability to stay protected by their niches, highly efficiency in repairing DNA damages) the discovery of innovative therapeutic approaches has become a priority to really improve the continuum of care concept of PC patients.

10.14 CSCs and Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most common and aggressive primary tumor of the central nervous system. Despite surgical resection and adjuvant radiochemotherapy, the median survival is approximately 15 months. Mounting evidence suggests that GBM arises from a rare cellular population which shares functional properties with neural stem cells (NSCs). Microarray analysis, in fact, revealed considerable similarity in gene-expression profiles between GBM and normal progenitor cells of the developing forebrain. Although little is known about the hierarchal organization of the brain, the evidence that NSCs are located in privileged areas, such as the subventricular zone (SVZ), makes plausible that these regions may represent the major source of GBM-initiating cells. GBM-initiating cells have been isolated from fresh specimens based on the expression of CD133 [35], a marker initially identified for NSCs. Like their normal counterpart, GBM-initiating cells possess, in vitro, the general characteristics of stem cells including extensive self-renewal and capability of multilineage differentiation. Most importantly, CD133+ cells are able to initiate a tumor in vivo after orthotopic transplantation in immunocompromised mice. However, a less clear distinction in the tumorigenic properties of CD133+ and CD133- cells has recently emerged [36]. In fact, since also cells that are devoid of CD133 can initiate a tumor, the presence of a yet uncharacterized, tumorigenic CD133- population is not so unlikely. The identification of tumorigenic GBM cells provided new insights into the mechanisms of resistance responsible for the failure of a multimodal treatment. CD133+ cells are not only resistant to radiation therapy but, moreover, their number increase after irradiation [37]. In fact, although CD133+ cells undergo DNA damage, these cells have a better ability to repair strand breaks via a more efficient activation of DNA-damage

checkpoints. Similarly, GBM-initiating cells are resistant to standard-of-care temozolomide. In this bleak landscape, however, innovative anticancer therapies are emerging. In a proof-of-principle experiment, for example, the pharmacologic inhibition of DNA-repair mechanisms, obtained by blocking Chk1 and Chk2 kinases, restored the sensitivity of CD133+ cells to ionizing radiation. Also the disruption of the vascular niche supporting GBM-initiating cells has emerged as a promising approach, as demonstrated by the growing number of clinical trials with the anti-VEGF monoclonal antibody bevacizumab and the pan-VEGFR tyrosine kinase inhibitor cediranib.

10.15 Eradication of CSCs

CSCs are responsible not only for tumor propagation, but are also the main actors in determining treatment failure. Thus, to eradicate a tumor, the priority is to exactly identify the target population. As discussed above, different cellular populations that share the hallmarks of CSCs often coexist in many tumors. In such a scenario, high-throughput genomic and proteomic technologies could provide powerful tools to capture a snapshot of key deregulated pathways in CSCs. If, in many cases, purification of CSCs is still limited by the lack of reliable markers, their detection and monitoring in vivo is an even more complex challenge. Far from being applicable in clinical practice, the development of new imaging techniques with single-cell or single compartment resolution, however, has made considerable breakthroughs in recent years. Thus, the measurement of the CSC compartment in cancer patients, for example by combining high-resolution imaging and CSC-specific probes, would allow the detection of early disseminated cancer cells, as well as a more accurate assessment of the radiologic response. Although resistant to current treatments, new insights into CSC biology have allowed a first wave of development of innovative drugs. As a result, different therapeutic strategies are on the horizon and include: (i) small molecules or monoclonal antibodies inhibiting stemness-associated pathways. Notably, some of these compounds are currently undergoing early phases of clinical development; (ii) agents able to restore sensitivity to chemotherapy and radiotherapy, such as those blocking ABC-transporters and DNA-repair mechanisms; (iii) pharmacologic strategies depriving CSCs of the necessary microenvironmental support, such as antiangiogenic agents and vascular disrupting agents, which impair the CSC-niche crosstalk; and (iv) differentiation-inducing agents. By way of summary, the acquisition of a more complete picture of pathways governing CSCs, combined with the progressive refinement of imaging techniques and the development of innovative pharmacologic associations, might pave the way for an effective eradication of CSCs.

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Appendix

The Impact on Surgical Practice of Recent Advances in Biotechnology. Interactions Between Inherited and Environmental Factors in the Occurrence - and Biological Behavior - of Diseases of Surgical Interest

F. Cetta

The recent advances in biotechnology (molecular biology, genome-wide association studies, proteomics) and in biomedical technology (CT scan, PET, radioimmunoguided procedures, minimally invasive surgery, robot assisted surgery) has dramatically affected the surgeon's decisions, involving both early diagnosis of diseases, multidisciplinary approach, multimodal treatment and also surgical technique and timing of surgical treatment.

Genetics, in addition to provide a precise diagnosis on the basis of the germ-line and somatic mutation, is also useful: (1) to improve our pathophysiologic knowledge; (2) to help us to select the proper treatment and the *timing* for treatment. This is of paramount importance in inherited multitumoral syndromes.

Lesson from Inherited Multitumoral Syndromes

In inherited multitumoral syndromes [1-19], in the presence of an individual with multiple different neoplasms – or at least bound to develop them in the near future – it is crucial to provide a list of the possible neoplasms which are typical of the syndrome and then potentially occurring during the subject's life, along with a scheduled list of treatments, and to select which is the most adequate treatment for that particular subject or when is the best time to do which.

In particular, it must be outlined that within the siblings belonging to a kindred with a given multitumoral syndrome, some neoplasms are *obliged*, i.e. they are bound to occur invariably in all siblings affected by the mutated gene, whereas other neoplasms show variable expression, i.e. they are organic to the syndrome,

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but their actual occurrence depends on interactions between the common germ-line mutation affecting the various siblings and epigenetic changes, which are usually induced by environmental factors [1].

The former group, i.e. obliged tumors, includes colonic polyps and colorectal cancers in patients with familial adenomatous polyposis (FAP) and germ-line mutation of the APC gene, mapped at chromosome 5 q 21, or medullary thyroid carcinoma, occurring invariably before age 5 in subjects with MEN 2A or 2B and germ line mutation in the RET gene, mapped at chromosome 10q 11. In particular, the precise site of the mutation in the RET gene (codon 619, 620, etc.) will dictate the biological behavior of the tumor.

Among the latter, i.e. those occurring only in some of the affected siblings and with a combination varying from individual to individual, we could include, in patients with FAP, papillary carcinoma of the thyroid [3-11] or brain tumors [15-18] such as medulloblastoma, but also desmoids tumors and other extracolonic manifestations, including hepatoblastoma and hepatocellular carcinoma [12-14,] ampulloma or pancreatic and bile tract tumors.

In particular, concerning both thyroid carcinoma and brain tumors, a striking female prevalence has been observed in FAP associated tumors, i.e. a F:M ratio of 50:1 in FAP associated papillary thyroid tumors, during the last 20 years, instead of 3:1 as occurs in sporadic tumors, or 18:0 as in the personal series of FAP PTC reported in Table 1, or a F:M ratio of 4:0, brain tumors (Tab. 2), instead of 1:2 with male prevalence as usually occurs in sporadic medulloblastomas.

Whereas colonic polyps and cancers occur homogenously in males and females [1, 19], this striking female prevalence in some extracolonic manifestations strongly suggests that the germ-line APC mutation plays a facilitating or predisposing role, but other factors, namely environmental factors, and likely factors associated with female sex, could also play an important role.

FAP associated PTCs and brain tumors are neoplasms which are integral to this multitumoral syndrome, because they occur only in patients with the specific APC germ-line mutation (and are more frequently associated with one germ-line mutation instead of another). However, additional factors are required for them to occur (environmental, dietary etc.). In addition they can be considered a typical example of interaction between genetic predisposition and environmental factors. In particular, since FAP-associated PTCs showed an increased incidence 5-10 years after the Chernobyl accident, it is possible that nuclear accidents could also determine long-term consequences, even in subjects living at long distance (thousands of km), in frail or genetically predisposed subjects [11].

Tumors integral to multitumoral syndromes are also likely to have a better biological behavior than their sporadic counterpart.

In particular, duodenal and/or pancreatic endocrine neoplasms associated with MEN1 showed a better survival and a reduced incidence of liver metastases than similar sporadic tumors, whereas breast cancers in subjects with BRAC1 and BRAC2 germ-line mutations were more frequently of the medullary histotypes, which is associated with a better prognosis. It has been suggested that patients with inherited germ-line mutations, facilitating the occurrence of multiple primary

Table 1 A personal series of cases comprising patients with FAP-associated papillary thyroid carcinoma (PTC)

Patient number	Sex	Age	Codon number	Exon number	CHRPE	LOH APC gene activation	Ret/PTC	BT
1	F	30	140	3	-	np	np	-
2	F	19	593	14	+	np	np	-
3	F	22	778	15	+	-	+	+
4	F	31	937	15	+	-	+	-
5	F	18	976	15	+	np	np	-
6	F	27	993	15	+	np	np	-
7	F	39	1105	15	+	np	np	-
8	F	34	1105	15	+	np	np	-
9	F	25	1068	15	+	-	+	-
10	F	26	1061	15	+	-	+	-
11[b]	F	22	1061	15	+	-	+	-
12[b]	F	20	1061	15	+	-	+	-
13[b]	F	36	1061	15	+	-	-	+
14	F	24	1061	15	+	-	-	-
15	F	20	1309	15	+	-	+	-
16	F	27	1309	15	na	np	np	-
17	F	22	na	15	na	np	np	-
18	F	20	na	na	na	np	np	-

APC, adenomatous polyposis coli; *BT,* brain tumor; *CHRP,* congenital hypertrophy of retinal pigment epithelium; *FAP*, familial adenomatous polyposis; *LOH*, loss of heterozygosity; *np*, not performed; *na*, not available

[a]In two patients, also with brain tumors

[b]Hepatoblastoma and hepatocellular carcinoma in a member of this kindred

Table 2 Brain tumors associated with PTC in the same patients or FAP kindred

Study	Sex	Age[a]	APC mutation	Brain tumor histotype	Patient age	PTC	CHRPE
Crail (1994)	M	24	1061	Medulloblastoma	24	+[b]	nr
Lynch (2001)	F	29	1061	Medulloblastoma	30	+[c]	nr
Fenton (2001)	F	29	1061	Medulloblastoma	6	+[b]	+
Plawski (2004)	nr	35	608	Cerebral flax tumor	na	+[c]	nr
	nr	10	608	Brain fibromatosis	10	+[c]	nr
Gadish (2006)	F	21	1061	Pinealoblastoma	18	+[c]	nr
Present series	F	22	778	Craniopharyngioma	16	+[b]	+
	F	36	1061	Medulloblastoma	32	+[c]	+
	F	20	1061	-	-	+[c]	+
	F	22	1061	-	-	+[c]	+

APC, adenomatous polyposis coli; *CHRP*, congenital hypertrophy of retinal pigment epithelium; *FAP*, familial adenomatous polyposis; *na*, not available; *nr*, not reported; *PTC*, papillary thyroid carcinoma

[a]Age (years) of first diagnosis of colonic polyps

[b]In the same patient

[c]In another member of the same kindred

tumors in different organs or districts stimulate a stronger immunological response (lymphocytic tumoral infiltration) which could be responsible for this more favorable biological behavior.

At any rate, it is noteworthy that even within the same kindred with the same germ-line mutation or within the same individual with an inherited multitumoral syndrome, environmental factors are of importance, both due to the occurrence of supplementary tumors, in addition to those *obliged*, i.e. occurring in all the subjects affected by the syndrome, and their aggressiveness.

Surgeons should become familiar with genetics and molecular biology of multitumoral syndromes, either for proper screening and early diagnosis of the various neoplasms occurring in each individual, or his/her siblings, or for the proper treatment and timing of treatment, which usually depend on the type of mutation and the biological behavior of these peculiar tumors.

Lesson from Inherited Multitumoral Syndromes Including Early Aging

It is likely that surgeons in the future will with increasing frequency be faced with individuals showing multiple primary solid tumors in diverse and not related organs and districts, even in the absence of inherited multitumoral syndromes. This is a consequence of the increased life expectancy and prolonged survival after diagnosis and treatment of the first malignancy [1, 11, 20-29].

Immunology and genetics suggest that the occurrence and progression of clinically evident malignancies is strictly related to aging and changes in mechanisms of the immune defense. In particular, some inherited syndromes, such as Werner's Syndrome and Rothmund-Thomson Syndrome, Bloom Syndrome and similar syndromes are characterized by early aging and greater predisposition to the occurrence of malignancies at an earlier age, in comparison with the rest of the population.

Interestingly, it has been suggested that in subjects with germ-line mutations of the genes responsible for these syndromes, environmental factors (air pollution, dietary factors) could determine a greater damage, with more severe complications in these frail predisposed individuals [1, 2, 11, 20-28].

In addition, it is well known that tumors occur or progress more rapidly or have a more severe behavior in immunodeficient or frail subjects. Their occurrence and development points to the break of the balance among noxious agents and host defenses. Subjects become *predisposed*, and it is more frequent and probable that a new tumor occurs in an individual who has previously been affected by other malignancies (Tab. 3).

Both multitumoral syndromes and multiple tumors in the absence of an evident inherited syndrome must be taken into account and could help proper detection of the new tumor. This is of basic importance, because early diagnosis is still one of the main criteria responsible for better treatment and increased survival.

Table 3 Personal series of patients with colorectal cancer associated with multiple different solid tumors in the absence of a detectable inherited multitumoral syndrome. All these patients had no clinical sign of colorectal malignancy. Cancer was detected by colonoscopy because of increased CEA values, after excluding recurrence

Age	Sex	Cancer and surgical procedures	Previous malignancies	Follow up
62	M	Ductal pancreatic carcinoma	Pancreatoduodenectomy	Alive (73y) and disease free (>11y after pancreatectomy)
64		Segment VII liver metastasis	Bisegmentectomy VI-VII	
68		Prostate carcinoma	no surgical treatment	
70		Colorectal (left colic angle) carcinoma	(Colectomy)	
40	F	Ductal breast cancer	Mastectomy	Death at age 68, because of colorectal metastases
45		Thyroid tumor	Thyroidectomy	(>9 years after hepatectomy)
60		Hilar cholangiocarcinoma	Left hepatectomy + CD lobe resection	
66		Colorectal (sigmoid) carcinoma		
49	F	Endometrial carcinoma	Hysterectomy with bilateral adnexectomy	Alive (69y) and disease free (>10y after hepatectomy)
59		Hilar cholangiocarcinoma	Right hepatectomy + CD lobe resection	
64		Right colon cancer	Colectomy	
58	M	Ampulloma	Pancreatoduodenectomy	Alive (69y) and disease free (>11y after pancreatectomy)
64		Left colon cancer	Colectomy	

Lesson from Hepatobiliary and Pancreatic Malignancies

Focusing on some hepatobiliary pancreatic tumors, it is well known that the 5-year survival after radical treatment of ductal carcinoma of the pancreas is only 10% and that in some patients with ductal adenocarcinoma disease free survival could also be greater than 10 years, i.e. the tumor can be considered clinically cured, as in some of our personal cases with pancreatic ductal carcinoma (or hilar cholangiocarcinoma) (See Tab. 3). In contrast, the remaining 90% of pancreatic cancers have a homogeneously dismal prognosis, with an average survival of 10-15 months, however aggressive and potentially radical the treatment. Extended lymphadenectomy and parenchymal resection have been invoked, but overall prognosis has little changed, despite the improvement of clinical skills and technology.

It is likely that among the apparently homogeneous group of subjects with ductal adenocarcinoma there is a great majority of subjects who will have a dismal prognosis, whatever treatment we provide. In contrast, there is a smaller minority that we are not yet able to identify in advance – because the tumor appear histologically similar – who will have long survival after radical treatment (Tab. 3).

The recently extended use of neoadjuvant chemotherapy in the multimodal treatment of pancreatic cancer facilitates the exclusion from radical surgical treatment of subjects with *no response* to preoperative chemotherapy. Even if we do not yet know why these patients do not respond, this preselection actually goes in the right direction of restricting aggressive and demanding surgical treatment only to those subjects with a more favorable biological behavior of the malignancy.

However, we have honestly to admit our current ignorance concerning a crucial issue, i.e. the timely detection of *which is which*, in order to reserve radical treatment and even extended and aggressive procedures to those subjects with more favorable tumors, and to select for palliative or low-risk treatments those with invariably dismal prognosis.

Lesson from Liver Regeneration

A better knowledge of pathophysiologic changes after liver resection has dramatically improved our approach to major liver surgery, in particular in patients with hilar cholangiocarcinoma. These subjects, usually severely jaundiced and septic because of previous endoscopic retrograde cholangiopancreatography (ERCP) [11, 23-26], are extremely frail and with reduced potential for liver regeneration after major liver resection, including more than 60% of liver parenchyma (trisegmentectomy, left or right, always including caudate lobe resection i.e. removal of segments I-VI- or IV-VIII plus I).

Therefore, in addition to procedures aimed at improving biliary stasis and cholangitis [25, 26], preoperative chemoembolization is usually performed of the

liver segments which are planned to be removed. This determines hypertrophy and improved function or residual segments, which could facilitate patient survival during the immediate postoperative period. In fact, after portal chemoembolization, liver volume of residual lobes increases of about 30%, usually within 4-5 weeks [27].

Pathophysiologic and oncological data show that:

- the strongest and most adequate stimulus facilitating liver regeneration and production of cytokines and growth factors is parenchymal trauma to the liver, in particular liver resection;
- liver regeneration, which usually occurs within 30-45 days after resection, involves all residual liver, with a *normal* regenerative response from normal parenchyma, but with tremendous hyperproliferative reaction from residual neoplastic tissue.

Therefore, removal of a single neoplastic nodule or multiple liver metastases, leaving in situ residual neoplastic nodules, must be considered with great caution in the absence of a planned multimodal treatment (e.g. removal of a small liver nodule to perform a correct diagnosis, when the primary tumor is still undetected).

In fact, in the presence of multiple liver nodules, these peculiar pathophysiologic consequences must be adequately taken into account, before planning liver resection, even using *minimally invasive surgical procedures*, if the possible side-effects of the procedure might include the *explosive* growth of residual tumors.

This is a typical example of how recent advances in biotechnology (molecular biology, genetics, a better knowledge of the role of growth factors in liver regeneration) should cope with advances in biomedical technologies (laparoscopic surgery or use of new therapeutic and diagnostic devices) in order to avoid severe side-effects and complications of the incorrect application of new technologies and provide the best therapeutic options for each individual.

Recent advancements in genetics and nanotechnology, in addition to being possible tools for therapeutic purposes, are also useful for a better knowledge of pathologic mechanisms and biological behavior of malignant (and non malignant) diseases.

In particular, in the near future it will be easier to distinguish between subjects with more favorable biological behavior and therefore susceptible to more aggressive and demanding surgical treatment and those who, regardless of an apparent radical surgical treatment, will have a dismal prognosis (e.g. 80-90% of subjects with ductal pancreatic carcinoma) [27].

Better therapeutic results are expected using an integrated multidisciplinary approach, combining both recent advances in biomedical technologies and a better knowledge of basic disciplines, including pathogenetic mechanisms and pathophysiologic linkages.

Lesson from Genetics and Genetic Engineering or Advances in Biotechnology: Surgical Implications

New drugs with molecular target (monoclonal antibodies against EGFR, VEGFR, anti c-kit drugs) are considered with increased interest and have shown to provide significant improvement in the treatment of patients with malignancies. However, even though we are still waiting for the *silver bullet*, i.e. a drug or device which is able to invert a pathologic pathway, and destroy or tackle selectively malignant cells with a low to nil impact on normal cells, it is self-evident that in the present context there is no chance for a single gene therapy, i.e. the possibility that the re-introduction of a wild-type gene in a subject with a mutated gene can switch the damaged pattern on again and enable complete tumor regression. In fact, before a clinically evident tumor occurs, not a single genetic mutation, but a long train of different genetic and biological alterations must develop, usually occurring during a long time lapse. It is therefore not realistic that a single drug or the reintroduction of a normal gene, instead of a previously altered one, or a new product of genetic engineering will be able to invert a long-term altered and chronically impaired mechanism responsible for a clinically evident malignancy.

In contrast, on the one hand while it is likely that new drugs, devices or treatments will be able to co-operate or be used together for a better multitumoral and multidisciplinary treatment of a single patient with single or multiple tumors, on the other improved biotechnology and the development of new devices and instruments or new technological advancements will also improve our present knowledge of basic pathophysiologic pathways and mechanisms which are responsible for the occurrence of malignancies and/or determine their severity or biological behavior.

In fact, thanks to further development in new technologies such as genome-wide arrays, proteomics and metabolomics, it is likely that in the near future we will be able to detect which is which, among various tumors affecting the same organ or district, and showing apparently the same histotype, but having a dramatically different prognosis. In other words, it may become possible to detect in advance which tumors have the best biological behavior, i.e. are less aggressive, more responsive to treatment, and therefore are going to have a better prognosis and yield an increased survival. This knowledge will dramatically affect the surgical approach, indications for surgery, technical procedures and the extension of planned resection, including the selection of a mini-invasive approach or, in contrast, justify an aggressive approach and extended resection, including major vessel resection and reconstruction, because of a *known favorable* biology and biological behavior of the tumor. Or, in case of unfavorable biological behavior, exclude surgical treatment in favor of palliative treatments.

Lesson from Pollution Related Diseases

Air pollution consists of tiny ambient particles measuring < 10–15 micron (PM10) and arising from dust, smoke, or aerosol liquids produced by vehicles, factories, or burning wood. In vitro studies have shown that exposure to diesel soot and other PM10 particles activates pro-inflammatory genes in a process mediated by free radical/oxidative stress mechanisms. These, in turn, induce pro-inflammatory transcription factors, such as nuclear factors-B (NFRB) and activator protein 1 (AP-1), which promote increased histone acetyl transferase activity, histone acetylation, release of interleukin 6 (IL-6) and interleukin-8 (IL-8), markers of inflammation, and, finally, expression of inflammatory genes [28-37].

The adverse health effects of air pollution are difficult to dissect since the atmosphere contains about 18,000 different substances, each of which is present at very low concentrations. Despite the well-known in vitro toxicity, mutagenicity, and carcinogenicity of many pollutants documented by experiments in animal models, it must be stressed that in most of these in vitro studies, the exposure level to each pollutant, e.g. polycyclic aromatic hydrocarbons (PAHs) and TCDD (dioxin), is higher than that occurring under actual conditions, in which PAHs are present at 10 parts per million (ppm), ozone at ppb (parts per billion), and TCDD at ppt (parts per trillion). Therefore the health damage caused by a single pollutant, even after long-term exposure, is likely to be very low.

In particular, airborne pollutants are currently considered weak pollutants. These are responsible for health effects which occur as a no-threshold phenomenon, namely that is no threshold above which all humans are affected and no threshold below which no effect is observable [31-33]. In other words, even very low concentrations of airborne pollutants (PM) can be responsible for health effects in particularly susceptible individuals [31-33].

Although this behavior has been confirmed both by epidemiological and experimental data, a linear dose-effect relation is still considered the main medianistinal linkage between concentration of pollutants and health effects. In other words, the greater the concentration of pollutant, the greater the number of hospital admissions.

In January 2008, a municipal fee (ECOPASS) was introduced in Milan to reduce the entrance of pollutant vehicles within the city. As a result, there has been an evident reduction in the vehicular traffic by up to 30-35% of private vehicles.

A scientific project called Prolife, involving all major local Universities, was started in January 2007, i.e. 1 year before the introduction of Ecopass. The project aimed at analyzing at 360° all the possible implications of health effects of air pollution, with particular reference to host-particle interactions in humans. The first step involved reproducing the same cross-sectional and longitudinal studies in Milan that have been performed in the United States and other countries.

In particular, during 2007-2009 we performed a comparison between the concentration of various pollutants and hospital admissions (53,514 hospital admissions because of cardiovascular and respiratory symptoms), and also longitudinal studies

in small cohorts of children attending primary schools (close to or far from main crossroads) and of old patients living in nursing homes, also comparing those living in hospices close to or far from main crossroads.

In particular, we performed 2 two-week campaigns per year, with mobile monitors placed either indoors and outdoors at the schools and nursing homes (corridors and gardens, respectively) and compared these direct field measurements with clinical findings, in particular in children, who all had a unique referral hospital. This made it possible to analyze any pollution related hospital admission and complications in children enrolled for longitudinal studies [33].

Lastly, the filters obtained during these first-hand seasonal campaigns, during which we measured both qualitative and quantitative parameters and for which we had the comparison with clinical findings, were used for in vitro studies.

Obviously, at the beginning, we reproduced previous in vitro studies using pulmonary alveolar cell lines (A549) or bronchial epithelial cell lines (BEAS-2B). However, after these preliminary studies, we also tried to make the same in vitro studies, with the same types of particles and the same variable concentrations, focusing on 2 distant districts, which on the basis of clinical and epidemiological findings could be affected by air pollution, namely sperm cells and synoviocytes [33-35].

In fact, it has been frequently reported that fertility is decreasing in males living in metropolitan areas, whereas rheumatoid arthritis is increasing, at least in females exposed to increased pollutant concentration, namely to traffic related pollutants.

The main reason for our choice was that both rheumatoid arthritis and male infertility – in particular in patients with varicocele – are two diseases in the occurrence of which autoinflammation plays a crucial role. In particular, the damage to sperm cells can be measured by semiquantitative methods, including both morphologic and functional parameters. This feature enabled us to make a quantitative comparison of the functional effects, not only varying the type and concentration of pollutants, but also varying the type of the host. In fact, we performed 3 different studies, using as host the rabbit, the normal human, and men affected by varicocele [33-35]. Data obtained suggested that, at least for some diseases, the variation of the host could be more important in terms of functional loss than the variation of the concentration or of the type of pollutants.

Lesson from In Vitro Studies of Airborne Pollutants on Synoviocytes and Sperm Cells

Oxidative stress is a working hypothesis that has been suggested as a common mechanistic linkage between particulate material and adverse health effects. But it is not unique. In particular, in a recent in vitro study in which different types of particles were used (PM < 2.5 or ≤ 10 μm/m^3 in aerodynamic diameter, tire debris), the same concentration of different particles with the same exposure time elicited different effects on sperm cell function (motility, viability, rate of apoptosis).

However, variability of the observed effects was less than that elicited by changing the host, with lower adverse effects in New Zealand rabbit sperm [34], more evident effects in human sperm, and very severe effects in humans with previous impaired sperm function (e.g. varicocele) [28]. In particular, sperm function could be reduced by up to 80% of the initial values in sperm cells from some humans with varicocele (unpublished data). Sperm cell function is easy to quantify and compare not only among different pollutants but also among different host species or subgroups [33, 34]. These findings are also in accordance with recent epidemiologic data showing more pronounced respiratory and cardiovascular effects in patients with previous respiratory and cardiovascular impairment or specific susceptibility, respectively [38].

Daily levels of PM_{10}, $PM_{2.5}$ and PM_1 were sampled and various concentration of PM ($10\mu g/cm^2$, $50\mu g/cm^2$, $70\mu g/cm^2$) were incubated at 37°C for 24, 48, 72 hours with synoviocytes from 5 patients with rheumatoid arthritis (RA) and 5 patients with osteoarthrosis (OA).

Synoviocyte-like fibroblasts (SLF) are cells of mesodermal origin that line the synovial cavity and are considered of importance in the pathogenesis of RA.

In particular: (1) PM was engulfed within synoviocytes; (2) after entering the cell, it determined an increased cytokine production (up to 9-fold the basal level of IL6) $p<0.001$); (3) this increased inflammatory reaction was more pronounced in synoviocytes from subjects with RA than in those from subjects with OA; (4) this 9-fold increase in IL-6 produced by cells which are distant from the portal of entrance of PM – and this is crucial for the occurrence of RA in humans – was greater than similar increases in cytokines, which had previously been observed by our group incubating PM, with cells from front-line districts (alveolar macrophages, A549 alveolar epithelial cell line; bronchial epithelial cell-line BEAS-1B [36].

These data show that SLF are able to determine an autonomous inflammatory response, which greatly also depends on the *intrinsic* properties and genetic imprinting of synoviocytes. Namely, those deriving from subjects with RA seem to be more inflammogenic than SLF from the control group (subjects with OA), thus suggesting that individual susceptibility, i.e. inherited predisposition associated with previous patient history and acquired susceptibility, plays a role greater than intrinsic toxicity of PM in the occurrence of RA [36].

Lesson from Asbestos Exposure and Mesothelioma in Humans

Data in accordance with these observations were also found in couples with the husband, who was occupationally exposed to asbestos for more than 30 years and who had not developed either asbestosis or mesothelioma, and the wife, who was exposed to asbestos only through her husband's clothes, who developed pleural mesothelioma. This lesson from asbestos exposure, together with recent results in patients with mesothelioma from Cappadocia [39, 40], suggests that individual susceptibility is of major important in the occurrence of asbestos related diseases [31-33].

In particular, a genome-wide analysis performed in a small subset of these couples (husband occupationally exposed, but unaffected, and wife unexposed but affected) showed a panel of differently expressed genes which were partly different from couple to couple, and partly common. Interestingly, common genes with diverse copy numbers included major histocompatibility genes, genes involved in the metabolism of xenobiotics and genes involved in the inflammatory response (Tab. 4).

Therefore, a great bulk of clinical and laboratory data suggests that the occurrence of clinically evident diseases is not simply related to the intrinsic toxicity of various pollutants. In fact, if intrinsic toxicity of pollutants were the main cause of health effects, diseases should be similar and homogeneously distributed in the various hosts. On the contrary, it seems that host-particle interactions generate health end-points, which greatly depend not only on individual susceptibility, but also on the type of the response and on the severity of the reaction, which are strictly related to previous patient history or immune habitus (RA vs OA) and also to tissue specificity [33-36].

In particular, we hypothesize that PM-related diseases are not simply determined by an inflammatory mechanism due to intrinsic toxicity of pollutants and mediated by oxidative stress as unique *common mechanism*, but more complex responses are generated, namely those typical of autoinflammatory and/or autoimmune diseases, which are more pronounced or clinically evident in predisposed subjects. And the degree of magnitude of the final clinical outcome can depend – at least for some diseases such as RA – more on host susceptibility and reactivity (host immune response) than on intrinsic toxicity or concentration of PM [33-36]. This will open new avenues for a basic role of autoimmunity in most PM related diseases. In particular, if PM actually reaches the synovial cavity, if autoinflammation is crucial for RA occurrence, and RA is part of the *pollution related syndrome*, then even low local concentration of PM could be sufficient to trigger pathologic events in predisposed individuals.

On the basis of the overall amount of available data we estimate that about 15-20% of the general population can be affected by a whatever disease linked with air pollution, even if susceptibility for each disease may be as low as 1%, whereas some individuals may be susceptible to multiple diseases in different organs or districts. Clinical experience shows that a greater proportion of susceptible children have non-susceptible parents. Therefore, in addition to inherited susceptibility, acquired susceptibility linked to environmental factors, including air pollution, plays an important role in the occurrence of this greater proportion of *susceptible individuals*. It has been proposed that, in particular for the respiratory tract, which reaches its final development after adolescence, exposure to air pollutants during the *perinatal susceptibility window* could be of major importance [36, 37].

There is increased evidence that children whose mothers have been exposed to increased concentrations of air pollutants during the third trimester of gestation or who have been exposed personally during the first months of life have a greater incidence of asthma at age 4 [37], i.e. there is a delayed effect, in comparison with the time of actual exposure. It is presumed that, during this high susceptibility window, even low proportions of pollutants may exert significant damage, i.e. the effect of

Table 4 Allelic imbalance analyzed by comparative genomic hybridization (peripheral blood) of the husband, who was occupationally exposed to asbestos for 30 years, without mesothelioma, and his wife, who was not occupationally exposed (only indirectly exposed through her husband) and who developed pleural mesothelioma at age 72. Nine genetic polymorphisms (7 with involved genes and 2 with no involved gene) were identified. list of genetic alterations (n=7) and involved genes

Mutation	Position	Gene	Protein	Function
1 Del 1q31.3	193513-193614 kB	CFHR1 CFHR4	Complement Factor H-related 1/4	Immunological response and lipid metabolism
2 Dup 4q13.2	69203-69311 kB	UGT 2B17	UDP-glucuronosyl-transferase (UGTs)	Conjugation and elimination of potential toxic xenobiotics
3 Dup 4p15.33	763-873 kB	ZTH HC11	Zinc-finger DHHC domain 11	
4 Del 6p21.32	32595-32660 kB	HLA-DRB5 HLA-DRB1	Major histocompatibility complex class II DR	Immune system regulator, by presenting peptides from extracellular proteins
5 Dup 8p11.22	39341-39449 kB	BCO 67864	AK128178	ADAM5 protein / hypothetical
6 Del 15q12	19869-19988 kB	OR4M2 OR4N4		Genes encoding for olfactory receptors
7 Del 15q14	32482-32751 kB	CR749361	Golgi autoantigen golgin-67	Golgi apparatus maintenance

this perinatal exposure can be amplified by one or more degrees of magnitude in comparison with later exposure of the same subject. Experimental studies also seem to support this view. In addition, since damage from xenobiotics during the perinatal period affects epigenetic mechanisms, previously unsusceptible subjects may become susceptible, and this acquired susceptibility may also be transmitted to future generations [37].

This mechanism, together with other mechanisms on pathogenic linkages, could be relevant for a better knowledge of how inherited factors (such as genetic alterations or germ-line mutations) may interact with environmental factors (diet, pollution, radiation), generating effects which could be restricted to the subject of interest, but which could also determine a permanent change in the genome or epigenome which is transmissible to offspring and which could be responsible for the occurrence of chronic or malignant diseases.

In particular, during the perinatal susceptible window weak pollutants or dietary toxicants which usually do not elicit severe or permanent alterations because of their very low concentrations could determine effects similar to those of in vitro studies that are usually performed with *pathologically relevant* concentrations, which are some degrees of magnitude greater than real world exposure. These mechanisms and these interactions should be better known not only by epidemiologists, statisticians and scientists involved in correlation studies, but also by surgeons, to be used in their everyday clinical practice.

In other words, a large proportion of the most critical effects in humans could occur because of exposure to increased pollution during perinatal life. The effects of this exposure may become evident many years later, as asthma at age 4, or in adult life as COPD or as tumors or cardiovascular diseases (or rheumatoid arthritis) in the elderly. Therefore, there will be an asynchronism between the time of exposure and the occurrence of clinically evident effects [36].

It may be possible, although this requires further documentation, that the greater bulk of future long-term effects (i.e. those related with acquired susceptibility) is actually due to exposure to pollutants during the first years of life. According to this hypothesis, even *pollution related lung cancer* (there are different types of lung cancers differently linked with smoke and/or air pollution) could be mainly determined during these first years, i.e. the exposure during these crucial years could play a greater role for the occurrence of future diseases than future exposure during later life.

These observations and inferences based on different pathophysiologic mechanisms greatly affect the results of epidemiologic studies comparing pollutant concentrations and concomitant hospital admissions. In fact, the latter are going to completely miss the health effects in newborns, which could be comparatively the most important [30-36].

Our working hypothesis [32, 33, 35, 36] is the following: usual concentrations of pollutants in most Western countries is responsible for very few and non-severe health effects in healthy humans. In contrast, the same pollutant concentrations could determine significant and severe effects in the elderly, or in children, or in frail predisposed individuals. Even among children and old people, clinical out-

comes will be evident only in a minority of the subpopulation. At the moment we are unable to distinguish which is which. A critical exposure will be possible in the perinatal period, during which the effect of pollutants could be amplified, and effects could be determined either in previously susceptible or in previously unsusceptible individuals, which will turn into susceptible because of this perinatal exposure. This is a crucial aspect of our hypothesis, which could explain the increased incidence of susceptible people during the last decades including those affected by nonmalignant diseases such as asthma, BPCO, cardiovascular diseases or male infertility, but also pollution associated malignancies [35, 36] (Figs. 1, 2).

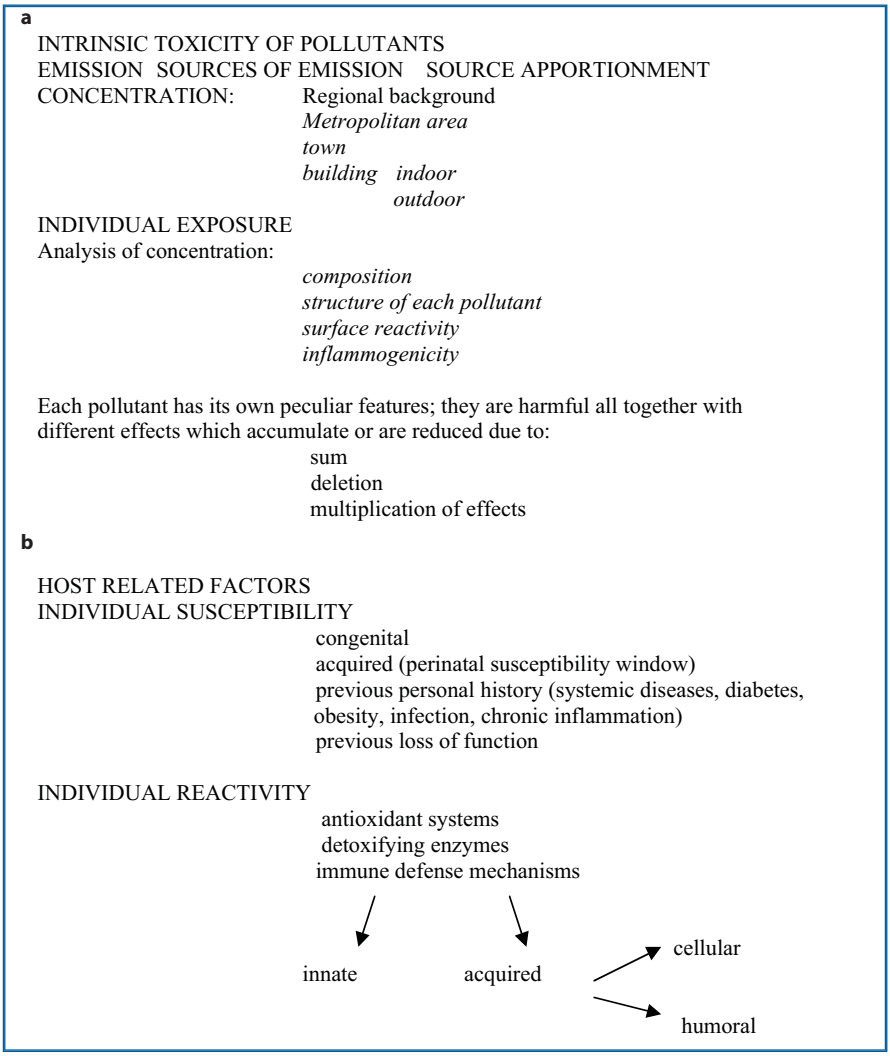

Fig. 1 Host-particle interaction depends on the particle side (concentration, composition, structure, surface reactivity of pollutants) (**a**) and on the host-side (individual susceptibility and reactivity) (**b**)

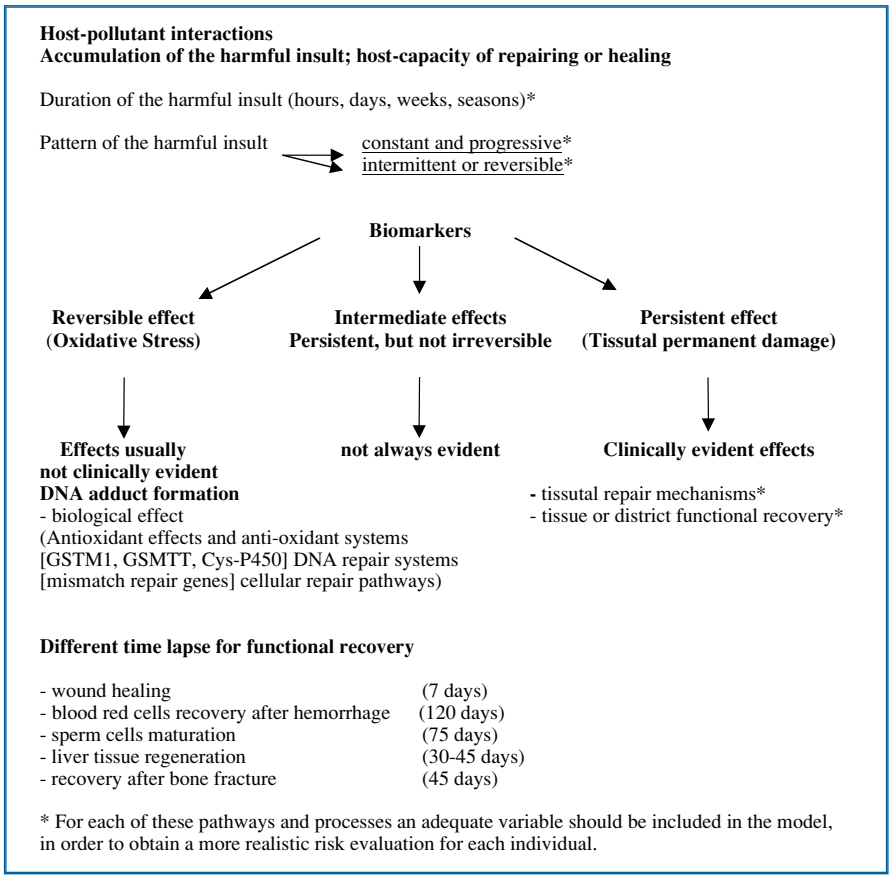

Fig. 2 Clinically relevant outcomes of host – particle interactions do not depend only on intrinsic toxicity of pollutants, but also on host- reactivity and repair ability of the host, which includes gene repair and cell function repair, but also tissue repair and functional recovery. Accordingly, biomarkers will include: markers of oxidative stress (reversible), intermediate effects (persistent) and of permanent tissue damage (irreversible). Only the last markers of irreversible effect are strictly related to final outcomes. The absence of reliable markers of persistent effect of PM is one possible factor of the lack of correlation between pollutant concentration and diseases responsible for hospital admission

Since individual susceptibility (or resistance) is crucial for the future occurrence of clinically evident diseases or symptoms, it is presumed that not a *pure toxic* mechanism will be responsible for clinical outcomes, i.e. a causative mechanism determining severe effects which are homogenously distributed in all individuals on the basis of the concentration of pollutants (linear dose-effect relation), but a different mechanism, involving more host related factors will be responsible for most final health effects (Fig. 3).

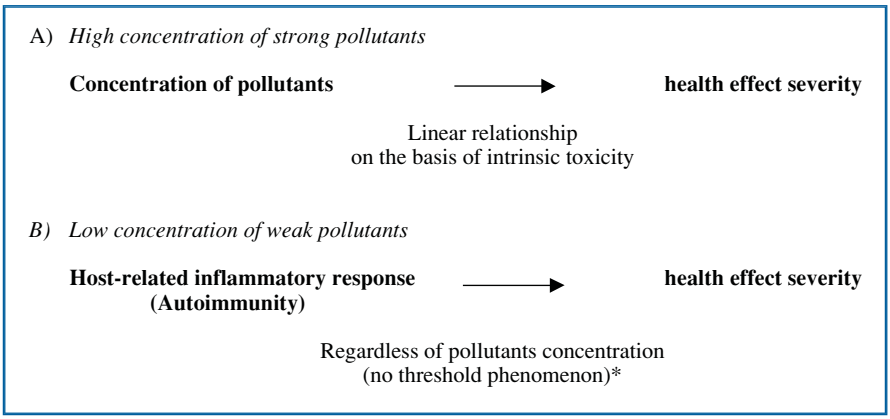

Fig. 3 Current paradigms to explain health effects of air pollution are mainly based on a linear dose and effect relationship (the greater the concentration of pollutant the greater the effect). An alternative pathogenic mechanism suggests that, at low concentration of weak pollutants, health effects could be due to host-related autoimmune mechanisms, relatively independent of pollutant concentration

Suggestions to Policy Makers to Tackle Health Effects from Air Pollution

If most pollution-related clinically evident diseases occur because of a major role of inherited or acquired factors (because of previous personal history or previous exposure), it may be more logical and more affordable as an objective to allocate resources into early recognition of susceptible at-risk individuals than to try to abate further PM concentration. The latter in densely populated metropolitan areas may appear as a non realistic goal, and also extremely expensive in terms of cost-benefits for human health.

Therefore, if the main goal of antipollution measures is not the simple reduction of measured air parameters, but a better treatment and/or a proper prevention of pollution related clinically evident diseases in humans, instead of fixing anachronistic and often difficult to reach objectives in metropolitan areas, where presence of humans and related activities (population density) are the most relevant causes of air pollution, a *sound* objective to reach could be the early detection of individuals at greater risk because of increased susceptibility, in order to electively address proper therapeutic and prophylactic measures for this subpopulation of at-risk subjects.

Lesson from Epidemiologic and Genome-wide Association Studies

Recently proper attention has been drawn to the rhetoric of false-positive results concerning environmental determinants and human health outcomes. In particular,

Boffetta et al. [41, 42] suggested that "users of epidemiological results outside the scientific community ... should be aware of the fact that statistically significant or positive results are often false" and that "epidemiology is particularly prone to the generation of false-positive results." In particular, in a review of 39 highly cited (citation index >1.000) randomized controlled trials that reported an original claim of an effect [43], only the results of 19 trials were replicated by subsequent studies. Therefore, caution should be applied in the communication of results to the media and the general public, because both tend to consider numbers and percentages as the *truth* and make their own speculations on data that are often based on inferences and weak associations [41].

However, the question of non-reproducibility of scientific results cannot be reduced to a mere controversy among epidemiologists – a controversy that should be limited strictly to them and treated only by improving statistical methods. It actually affects the basis of empirical knowledge, in particular when it involves biological and medical questions. When sensational new discoveries are counter to empirical observation, caution is mandatory. Biases may be detectable by epidemiologists, but there are other possible sources of basic errors concerning pathophysiologic mechanisms that are peculiar to each disease and that are unknown to statisticians, who apply the same methods to a wide variety of different conditions [2].

Biological plausibility is not enough. Individual susceptibility plays a role greater than previously supposed in the occurrence of clinical outcomes in the host due to environmental factors. The importance of susceptibility reflects a decreased relative role of pollutant concentration (i.e. intrinsic toxicity of xenobiotics [inhaled or ingested]), and reduces the applicability of certain models – based on dose and effect linearity – to no-threshold phenomena [2, 31-34] (Fig. 3).

Proper selection of subgroups, which should be homogeneous not only for age and sex but also for pathophysiologic relevance, is not only an epidemiologist's task but should be directed also by clinicians and pathologists. For example, lung cancer is still considered by epidemiologists as a single entity, but clinicians are aware that, in addition to cancer occurring in an anthracotic lung, pulmonary cancers may also occur in non-anthracotic lungs; this is a different disease less likely to be dependent on air pollution [2]. The knowledge of this fact will greatly affect population selection.

In particular, a senior clinician (surgeon, internist, clinical specialist) having long-term experience with the disease of concern should always be involved in the design of the study and in reporting study results. Interdisciplinary control of research is not only a desirable option, but a necessary measure to mitigate the sensational effect of new discoveries. This is true in particular when, despite statistical significance of observed differences, findings are counter to everyday clinical experience, or they are not clearly adherent to – or a logical consequence of – strict criteria such as Koch's postulates [1, 2]. Clinicians could also suggest the proper timing for large and expensive epidemiological trials, which should be performed exclusively when adequate metrics and reliable pathophysiologic causative mechanisms between determinants and outcomes have been established. Our view is that clinicians should be involved both in study design and timing, so that interdisciplinary control of the study can be guaranteed from the beginning [2].

Conclusions

The recent advance in biotechnology has dramatically changed not only the surgeon's approach to diagnosis and treatment of diseases of interest, but also his role in multidisciplinary research.

Concerning malignant diseases, the increased life expectancy that has been achieved with dramatic improvements in the diagnosis and treatment of primary tumors has been accompanied by the occurrence of second or third solid tumors in some cancer patients. These multiple tumors are apparently not related to germline mutations of tumor suppressor genes [1].

Increased exposure to traffic-related air pollution in densely populated metropolitan areas and to a wide variety of genotoxic xenobiotics introduced either by diet or by inhalation, together with spontaneous mutations related to aging are likely responsible not only for the observed incidence of chronic inflammatory diseases but also of malignant tumors. Future research will better elucidate the mutual, highly complex interconnections between inherited and environmental factors in the occurrence of malignancies. The phenotypic manifestations of the same germline mutation of a tumor suppressor gene are highly variable, even when patients belong to the same kindred. This is mainly due to superimposed epigenetic factors, which could be sex-based or environmentally related [1]. Likewise, health damage from occupational exposure to known carcinogens such as PAHs or even asbestos greatly varies among individuals with the same exposure level and/or belonging to the same family because of individual susceptibility [32-37, 44-46]. This includes inherited predisposition due to ethnic or individual differences not only in genetic polymorphisms for the genes encoding enzymes involved in xenobiotic metabolism, but also in *acquired predisposition*, related to the effects of aging, concomitant chronic or metabolic disease, such as infections, immunodepression or diabetes, and variable exposure to environmental agents, beginning from fetal development and/or the first weeks of life.

On the one hand, surgeons should be more prone to learning lessons from other disciplines, namely oncology, genetics, molecular biology, biotechnology and bioengineering in order to use these new technologies not only for better knowledge of the biological behavior of the various diseases, but also for the proper treatment and a multidisciplinary approach to new complex phenomena (for instance, multitumoral syndromes, or multiple primary tumors or metachronous tumors). On the other hand, surgeons, as well as all clinicians belonging to various subspecialties, should be involved from the beginning in the design of epidemiologic or correlation studies, which up to now have been considered the exclusive realm of geneticians, biologists, molecular biologists, epidemiologists, statisticians, concerning the degree of association between congenital factors or environmental factors (air pollution, dietary habits) and clinically evident diseases, including a better knowledge of mechanistic linkages and causative mechanisms. In fact, the latter should not only be biologically plausible on the basis of in vitro studies and studies in experimental animals, but should also be relevant, from a pathophysiologic point of view,

to clinical pictures and diseases observable in the real world. Otherwise, improper inferences can be made by epidemiologists or statisticians or biologists, who try to make uncorrected extrapolations from their data [41-43].

Therefore the surgeons and clinicians of the future, on the one hand should be more specialized in a smaller subset of their own discipline to guarantee state of the art treatment to their patients, while on the other they should be open to cooperate with other specialists, and play a primary role from the beginning in the design of epidemiological studies concerning the health effects or clinical outcomes of inherited germ-line mutations or environmental factors (such as air pollution, dietary habits), or aging or age related alterations, in order to avoid biased results and improper inferences, which otherwise are very frequent (up to 85%) [41-43] in the absence of deep involvement of clinicians in multidisciplinary studies.

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