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Cancer Biology and the Nuclear Envelope

Recent Advances May Elucidate Past Paradoxes

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This book is dedicated to the memory of Sir Kenneth and Noreen Murray whose devotion to science has been a major support for research at the University of Edinburgh.

Preface

It used to be said that "All roads lead to Damascus," and this was subsequently changed to "Rome." Today, it might be more appropriate to say "All roads lead to cancer." Half a century of focused modern research efforts have failed to find a "cure" for cancer because of the plethora of causes and mechanisms that can instigate tumorigenesis. Despite these many roads, the resultant tumor cells nonetheless share a handful of characteristics. To proliferate, cancer cells must have reactivated the cell cycle and often cell cycle regulators and signaling pathways that maintain a differentiated state are altered in tumors. Loss of genome integrity may or may not be causative in the progenitor cell, but it clearly becomes a characteristic within the tumor with chromosome translocations, DNA damage, and significant changes in transcriptional profiles all characteristic of pretty much all tumors. Moreover, the degree of metastasis is often correlated with the extent of DNA damage and chromosome translocations. Component cells of metastatic tumors migrate to spread and so cytoskeletal changes that enable cell migration are highly characteristic of more malignant tumors.

Even before any of the above-mentioned characteristics of tumors were identified, it was noted that most tumor cells exhibited changes in the shape and size of the nuclear envelope. Thus in the modern era as soon as the first nuclear envelope proteins were discovered—the nuclear lamins—they became a focus of research. Many correlations between lamin levels and increasing cancer grade were observed, and so lamin levels were added to nuclear size and shape changes in tumor diagnostics and prognostics. However, in some tumor types increased metastasis correlated with increases in certain lamins, while in other tumor types it correlated with decreases in the lamins. Therefore, the nuclear envelope was dropped as a major focus of cancer research.

In recent years, the nuclear envelope has been found to play important roles in cell cycle regulation and signaling, genome organization, the regulation of gene expression, DNA damage repair pathways and genome stability, and cytoskeletal organization, cell mechanical stability, and cell migration—all of the above noted

general characteristics of cancer cells. Many recent studies revisiting the nuclear envelope as a player in tumorigenesis and cancer metastasis have found cancer associations through the above-mentioned central mechanisms/characteristics as well as several unexpected links. On this basis alone it is clearly time to make the nuclear envelope a major focus of cancer research. However, there may be an even more compelling reason in recent findings that nuclear envelope protein composition is highly tissue specific. Indeed, with the many general cancer functions already linked to the nuclear envelope this finding could be the Rosetta Stone that explains much of the tissue/tumor type-specific aspects of cancer and the reason that in the early studies certain nuclear envelope characteristics correlated with increased metastasis in one direction or another based on the tumor type.

This volume brings together many different researchers and perspectives covering the historical and current use of the nuclear envelope in cancer diagnosis and grading, clear and potentially relevant functions of the nuclear envelope in cell cycle regulation and signaling, chromatin organization and gene expression, genome stability, nucleocytoplasmic transport, cell mechanical stability and migration, as well as unexpected links between the nuclear envelope and tumorigenesis. We have tried to collect some divergent viewpoints as well as representing both clinical and basic research and both facts and conceptual ideas. Our hope is that this collection will inspire new directions in cancer research as well as a new focus on the nuclear envelope. We now know that the nuclear envelope is as complex a signaling node as the plasma membrane and perhaps the next phrasing of that old quote will be “all roads lead to the nuclear envelope.”

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Contents

Part I
History and Use of the Nuclear Envelope in Cancer Prognosis

Introduction

Reports of differences in cell morphology in tumor cells go back to at least the mid-1800s, and many consider Sir Lionel Beale at this time to be the true father of cytology, when he described the aberrant morphology of cancer cells in various tumor types and ascribed a diagnostic and prognostic value to nuclear size and shape differences [1, 2]. Over 150 years later, nuclear size and shape are still used extensively in the clinic with clear statistical correlations having been observed in particular tumor types between nuclear size and shape defects and worse clinical outcomes. Eukaryotic cells tend to maintain a roughly constant ratio of nuclear to cell volume, the karyoplasmic ratio [3, 4], and changes in this nuclear size ratio are used as a prognostic indicator for the clinical outcome of various tumor types (e.g., [5, 6]). However, increased malignancy is linked to increased nuclear size for some tumor types, while it is linked to decreased nuclear size for other tumor types [7]. For example, increased nuclear volume is linked to malignancy for invasive meningiomas and bladder carcinoma [8, 9], while smaller nuclear volumes correlated with malignancy for squamous cell carcinoma of the lung [10]. In contrast, greater nuclear shape changes tend to always correlate with increased metastasis.

It would seem intuitive that the nuclear envelope is a nexus for such changes in nuclear size and shape, but this could not even begin to be tested until over 100 years later when the first nuclear envelope proteins were discovered. These were the lamins, among the most abundant proteins in the nucleus besides histones, at ~3 million copies per average mammalian nucleus [11]. There are three lamin genes, A, B1, and B2, and, of these lamin A was strongly reduced in certain cancers (e.g., [12, 13]). The subsequent finding that lamin A only appeared at later stages in differentiation [14] birthed the hypothesis that loss of lamin A reflected a dedifferentiation event in tumorigenesis [15]. However, it was soon noted that, in other tumor types, increases in lamin A expression, instead of decreases, correlated with worse clinical outcomes [16]. Other lamins have also been observed to change levels or

phosphorylation state in particular tumor types. For example lamin B1 is reduced in colon carcinomas, colon adenomas, and gastric cancers [17], while lamin B2 is hyperphosphorylated in leukemia [18].

Other nuclear envelope proteins besides lamins may play roles in nuclear size and shape changes in tumors, and these are covered in later sections of this book. Other sections also address the molecular mechanisms behind these changes and other cellular functions influenced by the nuclear envelope that when perturbed can lead to pathogenesis. This first section focuses on the historical and current clinical use of lamin levels, nuclear shape and size changes, and nuclear envelope markers to better detect nuclear shape and size changes in cancer diagnostics and prognostics. The first chapter is more of a short introduction, starting with the work of Professor Müller, Professor Bennett, and Dr Beale in the 1800s, focusing on the long history of using nuclear characteristics in cancer diagnosis and the technological developments that made this possible, and providing an overview of the nuclear envelope as a hub of connections to cancer biology. In the second chapter Jos Broers and Frans Ramaekers of Maastricht University, who have truly led the way for understanding differences in the individual lamin subtypes in different cancer types and tissues, present a beautifully detailed history of the use of expression levels of different lamin subtypes in cancer diagnosis and prognosis, starting right at the time that lamins themselves were discovered. Regulation of apoptosis is also critical in cancer pathology and, as a lamin A mutant intransigent to cleavage delayed apoptosis [19], the role of lamins in apoptosis and its relation to cancer are also discussed. The remaining chapters in this section are contributed by three clinical world leaders who are studying and perfecting the use of the nuclear envelope in cancer diagnosis and prognosis. In the third chapter Andrew (Andy) Fisher from the University of Massachusetts Memorial Medical Center gives a delightful discussion of the value of different characteristics of the nuclear envelope including size, chromothripsis, and various types of shape changes in cancer prognostics. He presents a very insightful view on the appropriate grouping and weighting of these parameters as well as theories on how they reflect the processes of tumorigenesis and malignancy. In the fourth chapter Robert (Bob) Veltri and Christhunesa Christudass of Johns Hopkins Hospital delve into the history of the modern fusion between microscope and computer in developing methods to evaluate nuclear morphometry and applying this to clinical grading of prostate tumors for optimizing treatment. Their chapter brilliantly conveys the practical aspects of quantifying nuclear envelope differences in cancer pathology. The fifth and last chapter in this section by Gianni Bussolati and colleagues from the University of Turin pushes for changes in the methods used for assessing nuclear shape differences. They clearly demonstrate that enormous improvements in resolution are obtained when staining for nuclear envelope markers by immunofluorescence compared to standard approaches of hematoxylin and eosin staining [20]. This new approach enables different thyroid cancers and diseases to be distinguished based on biopsy that could not be before and

increases the confidence of clinical grading for breast cancer. Together these chapters provide a solid overview and discussion of the existing methods and future directions in using the nuclear envelope for cancer grading and prognostics.

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The Nuclear Envelope and Cancer: A Diagnostic Perspective and Historical Overview

Jose I. de las Heras and Eric C. Schirmer

Abstract Cancer has been diagnosed for millennia, but its cellular nature only began to be understood in the mid-nineteenth century when advances in microscopy allowed detailed specimen observations. It was soon noted that cancer cells often possessed nuclei that were altered in size and/or shape. This became an important criterion for cancer diagnosis that continues to be used today. The mechanisms linking nuclear abnormalities and cancer only started to be understood in the second half of the twentieth century, with the discovery of nuclear lamina composition differences in cancer cells compared to normal cells. The nuclear envelope, rather than providing a mere physical barrier between the genetic material in the nucleus and the cytoplasm, is a very important functional hub for many cellular processes. In this review we give an overview of the links between cancer biology and nuclear envelope, from the early days of microscopy until the present day's understanding of some of the molecular mechanisms behind those links.

Keywords Cytology • Diagnostics • Karyoplasmic ratio • NETs • NPC • Nuclear lamina • Nuclear size

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Abbreviations

H&E Hematoxylin and eosin
NET Nuclear envelope transmembrane protein
NPC Nuclear pore complex

The Nature of Cancer: From Ancient Egypt Until the Early Twentieth Century

We often talk about efforts to cure cancer as if they had only been going on for the past 60 years or so, but several papyri dating from roughly 2000 to 1500 BC indicate that the ancient Egyptians were able to distinguish between benign and malignant tumors and described the surgical removal of tumors, cauterization, and pharmacological as well as magical treatments for the disease [1]. Hippocrates (460–370 BC), father of Western medicine, used the word karkinos (crab) to name the disease that he described as producing hard swellings that were of a noninflammatory nature and had a tendency to spread through the body, causing death. At the time, all diseases were attributed to an imbalance in the body's four elemental humors: blood, phlegm, yellow bile, and black bile. The humoralist theory remained popular until the mid-1800s, when the cellular nature of cancer was identified. The reason for this change in attitude is simply a technical one: the improvement in the microscope's optics allowed much more detailed examination of specimens.

Microscopy was well established and used in biology for nearly 200 years before it became of assistance to cancer biology [2]. However, early microscopes suffered from chromatic and spherical aberrations that made detailed observations difficult. The modern microscope was born when the English physicist Joseph Lister (1786–1869) showed that spherical aberration could be minimized by a careful combination of lenses. He published his work in 1830 [3], and by the 1840s his microscope was used widely around the world. This microscope represented a significant improvement over previous models, bringing down the resolution to about 1 μm. Improved optics and development of differential staining techniques facilitated the examination of cancer cells (as well as from other pathologies) with a degree of detail unimaginable merely decades earlier. It was soon recognized that microscopic study of pathological specimens provided a very useful tool for the diagnosis of diseases, including cancer.

In the early 1890s the German zoologist Theodor Boveri recognized the genetic basis of cancer [4]. Boveri is principally credited with the discovery of chromosome territories, but he made some of the biggest and most significant leaps in cancer theory in history. He postulated that chromosomes were distinct from each other and transmitted heritable traits. He suggested that chromosome mutations could give rise to a cell with the ability to grow without limits and that this cell could pass on this ability to its descendants. He also proposed that there could be checkpoints, tumor-suppressor genes and oncogenes, and that cancers could be caused by radiation, physical or chemical insults, or pathogenic microorganisms.

The Early Observations of Cancer Cells

Although cancer had been diagnosed as a disease and studied for at least four millennia, its diagnosis remained relatively basic, with no significant advancement in understanding until the mid-1800s. Suddenly, improvements in microscopy led to a flurry of activity between the late 1830s and the 1860s that completely changed modern medicine and its attitude to cancer.

The German scientist Johannes Peter Müller (1801–1858) is considered to be the father of medical microscopy and pioneer of clinical cytology. In his 1838 "Über den feineren Bau und die Formen der krankhaften Geschwülste" (which translates as "On the Nature and Structural Characteristics of Cancer, and of Those Morbid Growths Which May Be Confounded with It") he was the first to describe cancer cells in detail and to note how they lose adherence when compared to normal cells [5]. Based on the physical characteristics he observed, such as altered cell morphology, reduced cell adherence, and altered tumor mass rigidity compared to the surrounding tissue, Müller developed criteria to diagnose benign and malignant neoplasms as well as to distinguish between sarcomas (tumors with abundant connective tissue) and carcinomas (tumors with little or no connective tissue). He ran a state-of-the-art laboratory at the Humboldt University in Berlin, with the best microscopes of the day that could resolve down to 1 μm. Many of his assistants became prominent microscopists themselves: these included Friedrich Henle who developed the early germ theory of disease, Robert Koch who founded the field of bacteriology and received the Nobel Prize in Physiology or Medicine in 1905 for his work on bacterial pathogens, Theodor Schwann who developed the cell theory, and Rudolf Virchow who built on Schwann's work and became the father of modern pathology, rejecting the notion of spontaneous generation with his "omnis cellula e cellula" (which can be translated as "every cell comes from another cell") and bringing an end to the humoralist theory of human disease that had been prevalent for the previous 2,000 years.

Müller's monograph in 1838 appears to have had the effect of turning the attention of physicians and scientists sharply on to cancer. In the next few years, several very important scientific articles were published that marked the path for pathological cytology.

Illustrations in scientific journals during most of the nineteenth century consisted generally of drawings carved in wood blocks that were subsequently stained and used to print the illustrations. In the 1840s the French physician Alfred Francois Donne (1801–1878) was the first person to apply photography to microscopy. He invented the photoelectric microscope, which enabled the projection of microscopy images onto a wall. These projections could then be captured as a daguerreotype, an early form of photography. In 1844 he published his "Cours de Microscopie Complementaire des Etudes Medicales," the first atlas of microscopic anatomy, illustrated with numerous photographs [6]. Donne was the first to describe leukemia and show photographs of blood cells from both autopsy specimens and living patients. The following year, in 1845, leukemia was recognized as a blood disorder by the English physician John Hughes Bennett (who had been a student of Donne's),

in Edinburgh [7]. Microphotography did not become popular until nearly 50 years later, and despite Bennett's relationship with Donne, his publications only contained relatively basic drawings.

The first detailed and comprehensive description of the altered morphologies of cancer cells, as well as tumor anatomy and the different behavior of cancers in a variety of organs, came from the Irish physician and Edinburgh University graduate Walter Hayle Walshe (1812–1892) in 1846 [8]. His work is also one of the earliest examples of statistical analysis of cancer frequency according to age and gender, looking at lung cancer, which was already by then recognized as one of the most common forms of cancer. Unfortunately, despite the great detail of description, Walshe included no illustrations in his work, thus limiting its impact and utility to train other physicians.

A year later, in 1847, the physician Julius Vogel, a disciple of Müller, published his pioneering book on pathological anatomy [9]. He was one of the first to diagnose cancer using a method that later became known as exfoliative cytology (the microscopic examination of cells that are shed with a gentle scrape from various surfaces of the body, such as the inside of the mouth), rediscovered and brought to the fore by George Papanicolaou 80 years later, in the early twentieth century.

Then, in 1849, Professor Bennett published "On cancerous and cancroid growths" where he described cancers of a variety of organs [10]. In this work Bennett experimented using acetic acid treatment to aid the visualization of specimens, in which he noted cancer cell polymorphism and presence of multinucleated cells as well as cells with an increased number of nucleoli, which we now know to be a reflection of the increased ploidy level that is frequently observed in cancer cells (Fig. 1). This work was published with the publishers advertizing "190 illustrations, copied from nature, and drawn on wood by the author."

In 1851, Hermann Lebert (1813–1878) published a treatise [11] where he described the characteristics of malignant cells, their variation of sizes, and noted the commonly increased size of the nucleus compared to the cytoplasm (later known as the "karyoplasmic ratio" [12]). This is the first description of altered karyoplasmic ratios in cancer cells. Alteration of karyoplasmic ratios is a morphometric criterion still used today in diagnostics, well over 100 years later, and is only now beginning to be understood.

By the early 1850s, barely over a decade after Müller's monograph, the literature on cancer anatomy and pathology had multiplied and commonly included very useful—if still a bit crude—drawings of cancer cells. This was in great part due to the rapid advances in light microscopy that took place in those days. However, the microscopes were not easy to use and without stains to aid visualization, diagnosis remained a difficult and time-consuming task, as Lebert had noted in 1845 [13].

Sir Lionel Smith Beale (1828–1906) was an English physician and microscopist at King's College in London and is now considered the true father of cytology. He learnt from Professor Bennett that some acid or alkali treatments of specimens resulted in differential staining of cells. He further developed the differential staining technique to improve microscopic observations, noticing that active nuclei stain intensely using basic dyes whereas dead cells could be stained with acid dyes. In 1854 Beale published "The microscope and its application to practical medicine" [14].

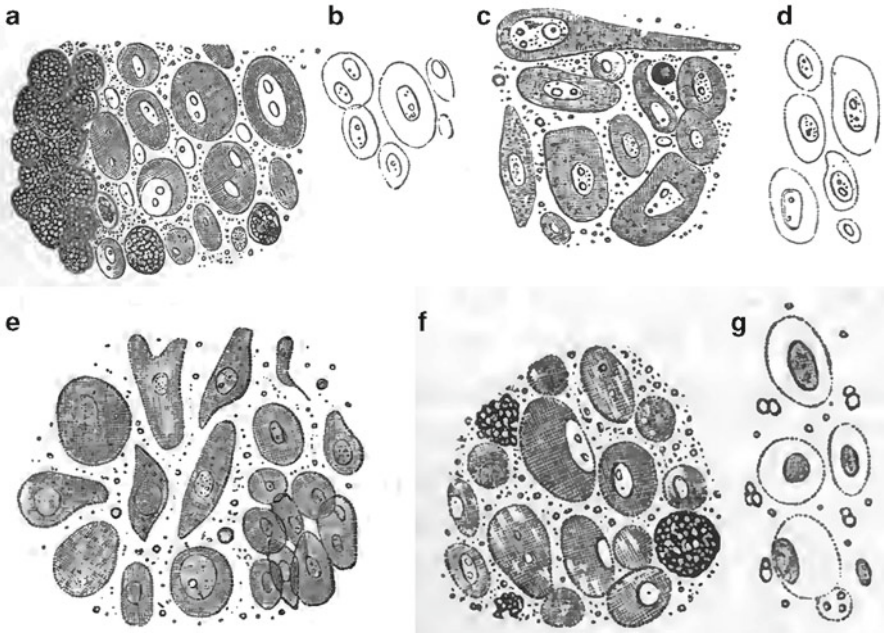

Fig. 1 Cell and nucleus size polymorphism in cancer cells. Adapted from Bennett [10]. (**a**) Cancer cells from a breast tumor, showing cellular and nuclear size polymorphism. (**b**) Same sample as (**a**), after treatment with acetic acid, which renders cytoplasm partially transparent. (**c**) Cells from a recurrent breast tumor, from a different individual than (**a**). (**d**) Same as (**c**), after treatment with acetic acid. (**e**) Uterine cancer cells, with cell and nuclear size and shape polymorphism. (**f**) Cancer cells from a liver tumor. (**g**) Same sample as (**f**), after treatment with acetic acid

In the first part of this volume Beale describes various types of microscopes available at the time and staining techniques that can be used to improve the visualization of clinical specimens. In the second part of the volume he describes a wide range of pathologies, diagnosis, and treatments and includes many illustrations of microscopic observations. In particular, he goes on to describe cancer cells of a variety of tumors, noting as diagnostic features the differences in their cell sizes and shapes, number and sizes of nuclei, and loss of adherence to adjacent cells in the biopsies. He discussed in detail ways in which cancerous cells could be distinguished from benign growths that may have a similar clinical appearance in a variety of tissues (Fig. 2). On the surface, these observations are not very different from those that Müller had noted and published 16 years earlier. What made Beale's work stand out was the quality of his illustrations and descriptions. His drawing abilities coupled to the use of basic specimen preparation and staining techniques meant that he was able to demonstrate with clarity what he saw under the microscope. In 1860, Beale published his now classic illustration of cells from sputum from a patient with pharyngeal cancer [15] (Fig. 3). His drawings were of such quality that a diagnosis can be derived from them today: the prominent cytologist Bernard Naylor stated

Cancerous.	*Cancroid.*
Cells not connected with the matrix in a regular manner, or forming laminæ.	Cells connected with the matrix, often forming distinct laminæ.
Cells differing much from each other in size and form.	Cells resembling each other in size and general outline.
Cells readily separable from each other.	Cells often cohering by their edges, which generally form straight lines; three or four cells being frequently found united together.
Cells not connected together at their margins; their edges seldom forming straight lines.	
Cells containing several smaller cells in their interior often met with.	Cells usually containing one nucleus.
Nuclei varying much in size and number in different cells.	Nuclei not varying much in size in different cells.
Juice scraped from the cut surface containing many cells floating freely in the fluid, and not connected with each other.	Juice scraped from the cut surface containing small collections of cells, which are often connected with each other.

Fig. 2 Epithelial cancer cells, and diagnostic criteria to distinguish between malignant (cancerous) and benign (cancroid) growths. Adapted from Beale [14]

about this illustration: "It is obvious to us today that the patient died of keratinizing squamous cell carcinoma" [16]. Although Lionel Beale's work was perhaps not the most important in volume, he clarified the importance of cytological diagnosis and effectively communicated this to the rest of the scientific community. One of his most prominent supporters was Rudolf Virchow, whose greatest achievements were in microscopic pathology. Virchow published several major pathology textbooks, including "Cellular Pathology" in 1858 and a three-part series on tumors in 1863–1865 [17–20].

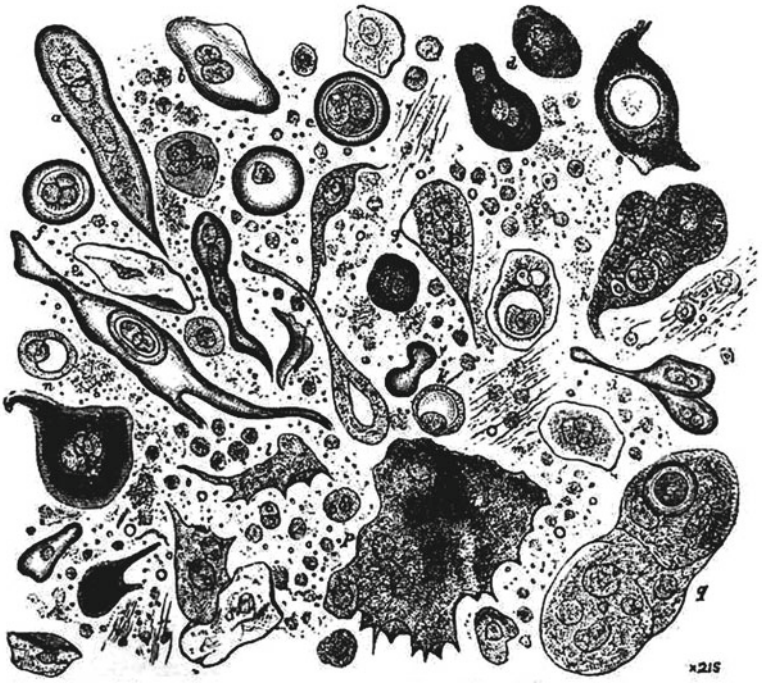

Fig. 3 Cancer cells in a sputum sample from a patient with cancer of the larynx. From Beale [15]

During the rest of the nineteenth century and early twentieth century, the advances in cancer diagnostics were mostly due to the development of specimen treatment techniques, such as formaldehyde fixation of tissues, and of novel stains, which helped physicians all over the world to publish their observations, as well as the development of microphotography. One of the most notable advances in staining was the development of the hematoxylin and eosin (H&E) stain in 1876 by A. Wissowzky [21], which is still in wide use today. With this method the nuclei are overstained dark blue in alum mordanted hematoxylin, followed by destain in dilute acid alcohol and blue color developing in slightly alkaline water. The cytoplasm is then stained orange-pink with eosin. H&E staining remains the gold standard for diagnosis of many cancer types.

The advances in cancer diagnosis developed in the mid-1800s resulted in the general public becoming more aware of cancer as a disease. Moreover, the increasing number of cancer diagnoses resulted in the perception of cancer as a rapidly growing disease and some degree of public fear. That the advances in diagnosis were not coupled with advances in treatment also gave the term cancer and its diagnosis the appearance of a death shroud, as can clearly be observed in the literature of the period. In response to this rising public fear and ignorance concerning cancer special research agencies dedicated to the investigation, education, care, and eradication of cancer were instigated in both the UK and the USA in the early 1900s.

The Early Modern Era of Cancer Diagnostics

Cytology as a scientific discipline developed and flourished in the twentieth century. The modern era of cytological pathology started with George Papanicolaou (1883–1962), working at the Anatomy Department of Cornell University, New York. In his 1928 paper "New cancer diagnosis" he proposed using vaginal smears to detect uterine cancer, using a polychromatic stain technique [22]. Papanicolaou described cancer diagnosis using cells gently scraped from the cervix of the uterus, based on a combination of changes in staining, size, shape, and characteristics of nuclear chromatin, assigning a numeric grade to each sample based on these parameters. This paper, a true hallmark of cancer diagnosis, was not received with much interest initially. Many pathologists were sceptical about the ability to diagnose cancer from scraped cells, when one of the most important features of cancer is tissue invasion, which cannot be inferred from loosened cells. Eleven years later, in 1939, Joseph Hinsey became the new director of the Anatomy Department and together with Henricus Stander, the director of the Gynecology Department, encouraged Papanicolaou to pursue his cancer research full time. The importance of Papanicolaou's work did not go unnoticed the second time, publishing mostly the same results in his commonly referenced 1942 Science article and two more papers written together with Herbert Traut [23–25]. Papanicolaou's smear test became known as the "Pap test" with its usage spreading rapidly during the 1940s, arriving in Europe after the end of World War II and becoming established as a routine check for uterine and cervical cancer. As a result of the establishment of such routine checks, cervical cancer mortality has greatly decreased from being the leading cause to the eighth most common cause of death from cancer in women [26].

Pap staining is not only used for uterine and cervical cytology. It was quickly discovered that it could be used for oral specimens [27, 28], and today it is used for a wide range of specimens, such as urine samples, cerebrospinal fluid, abdominal fluid, synovial and pleural fluid, fine needle aspiration biopsies, and many others.

The reason the Pap staining was such a success is that it retains nuclear detail and definition and cytoplasmic transparency and can indicate cellular differentiation of squamous epithelium. It is a polychromatic staining method that depends on the degree of cell maturity and metabolism, resulting in very detailed and distinct cellular staining. The basic Pap stain is derived from the classic H&E but contains several other ingredients:

1. Hematoxylin: Stains cell nuclei and allows a coarse observation of chromatin compaction.
2. Orange G: Stains keratin effectively. It stains small cells of keratinizing squamous cell carcinoma that may be present in sputum and other samples. The counterstain Orange G is high in alcohol and provides cytoplasmic transparency, enabling clear visualization of overlapping cells.
3. Eosin Y: Stains in pink superficial epithelial squamous cells, nucleoli, cilia, and red blood cells.
4. Acid Green: Stains cytoplasm.
5. Bismarck Brown Y: Stains cartilage and is nowadays often omitted.

The Late Modern Era: Automation and Computer-Assisted Image Analysis

The proper recognition of normal and cancerous cells is fundamental to diagnostic cytopathology, but the morphology of normal cells can vary greatly, depending on the tissue, and this can overlap with features of cancer cells. There is normally a continuum in the tissue variability. Diagnosis becomes critically dependent on both the availability of a marker for "abnormality" and the recognition of what is normal, typically by the eye of a well-trained pathologist.

The cytopathologist Stanley Patten (1924–1997) was one of the pioneers in the field of automation of diagnostic methods using a slit-scan cytofluorometer. Patten's initial interest centered around standardizing morphometric measurements of diagnostic potential to better define pathology and establish reliable and reproducible diagnostic criteria [29, 30]. George Wied (1921–2004), a disciple of Papanicolaou, also worked towards a standardization of cytologic terminology and morphological measurements, using acridine orange-stained material to obtain fluorescence intensity measurements that could be used to objectively calculate sample metrics [31–34]. With Wied and Patten the field of quantitative cytology was born. The morphometry parameters used include nuclear size, karyoplasmic ratio, and nuclear contour shape. Because microscope-based diagnosis is a demanding yet tedious task, the idea of automating screening of cervical smears and other samples soon arose. Wied was very interested in the possibility of automating sample analysis, but in the 1950s and 1960s computers were not yet widely used and were of minimal computing power. Despite that, by the late 1960s Wied had established a program to acquire and process cytological data. In 1970, his TICAS-MLD device was able to analyze cytological samples and produce an output with various cellular parameters that used clinical probability data for diagnosis [35]. As computing power and robotics rapidly increased in subsequent decades, full automation became possible, allowing the analysis of much larger samples for increased statistical power.

Wied and Patten are the pioneers in the field of automated diagnostics.

Today the work they started continues in the exciting research of clinicians such as Dr. Bob Veltri at the Johns Hopkins Hospital in Baltimore, Professor Gianni Bussolati at the University of Turin, and Professor Andy Fischer at the University of Massachusetts. Bob Veltri's team patented and commercialized, in 1996, the first statistical based algorithm to predict prostate cancer postoperative stage based on pretreatment biopsy data and quantitative digital image analysis. Professor Bussolati's laboratory has developed a cell nucleus 3D-reconstruction image analysis system, using the nuclear envelope protein emerin, to greatly aid the diagnosis of papillary thyroid carcinoma and breast cancer. Besides his interest in the molecular aspects of cancer diagnosis, Andy Fischer has invented the Cellient Automated Cell Block System, which automatically recovers small tissue fragments from a specimen container, using an improved microbiopsy needle, and delivers them rapidly to an indexable plane in paraffin for histologic sectioning.

These automated and/or computer-assisted diagnostic protocols outperform standard diagnostic procedures by pathologists in certain situations. It is interesting

that the diagnostic parameters employed are still largely morphological and nucleus centric, essentially the same type of features that cytologists have been looking at for the past 160 years.

The Use of Nuclear Morphometry in Cancer Diagnosis

Cytopathologists have long been using nuclear morphology alterations in cancer cells for diagnostic and prognostic purposes. Nuclear size changes, in particular, have a great diagnostic value for many cancer types. Tumor cells were often observed to have enlarged nuclei, although in a few cases the opposite is true and a reduction of nuclear size correlates with a worse prognosis (Table 1).

However, nuclear size observations alone are not enough for a reliable diagnostic. For example, in osteosarcoma a reduction in nuclear size is an indicator for poor prognosis, but only if accompanied by a reduction of the round appearance of the nucleus [53]. In general, cancer is diagnosed by a pathologist using a combination of morphological features. Nuclear size is only one of the nuclear metrics used in cancer diagnosis. There are other visible nuclear changes that the trained eye of the cytopathologist can use to diagnose, classify, and even differentiate between tumor types with different prognoses. Principal among these are the karyoplasmic ratio, nuclear roundness, nuclear envelope smoothness, chromatin distribution as

Table 1 Nuclear size alteration correlates with grade and poorer prognosis in many cancer types

Cancer type	Nuclear size change	References
Breast cancer	+	[36–38]
Male breast cancer	+	[39]
Cervical cancer	+	[40, 41]
Small-cell cervical carcinoma	+	[42]
Colorectal cancer	+	[43]
Epidermal squamous carcinoma	+	[44]
Cutaneous soft tissue sarcoma	+	[45]
Gastric carcinoma	+	[46]
Lung squamous cell carcinoma	–	[47]
Liver cancer	+	[48]
Melanoma	+	[49, 50]
Invasive meningioma	+	[51]
Oral squamous carcinoma	+	[52]
Osteosarcoma	–	[53]
Ovarian cancer	+	[54]
Pancreatic cancer	+	[55]
Prostate adenocarcinoma	+	[56]
Papillary thyroid carcinoma	+	[57]
Urinary bladder carcinoma	+	[58–60]

In most cases, an enlargement of the nucleus is associated with worse prognosis. The "+" symbol denotes nuclear enlargement in cancer, and conversely, the "–" symbol denotes nuclear size reduction

visualized with hematoxylin and other stains, and presence of nuclear envelope invaginations and grooves.

Though it is often difficult to pinpoint the original cause of a tumor because of the myriad of changes that occur, one general feature is that faulty control of cellular growth allows a particular "rogue" cell to proliferate in situations where it should not normally proliferate and which often develops the ability to invade surrounding tissue and ultimately migrate—metastasize—to other tissues. The genetics of cancer have been the focus of intense research for the past several decades. Tumor-suppressor genes, a class of genes that restrict cell proliferation, are often mutated or epigenetically silenced in cancer. Oncogenes can be abnormally activated, promoting cellular division. Mutations in checkpoint genes can allow a damaged cell to escape apoptosis and to continue to proliferate. DNA repair pathways can be impaired and promote further mutations and genome instability. However, despite all we have learned about the many mechanisms behind cancer, invariably a cytopathologist still makes the official diagnosis based on microscopic observations of biopsy material that are principally focused on nuclear morphological features.

Why is the nuclear envelope so good at diagnosis and predicting clinical outcomes for cancer? Francis Crick is alleged to have said: "If you can't study function, study structure." There are many structural ways that nuclear shape and size could provide tumor cells with an advantage in cancer.

The fact that very different cancers can arise by a variety of mechanisms and originate in different tissues, yet they tend to share a substantial number of the nuclear abnormalities mentioned earlier, suggests that these structural alterations have a significant functional consequence. The structure of the nuclear envelope is that of a double-membrane system with two completely separate lipid bilayers separated by a relatively uniform luminal space of ~50 nm in human cells. The two membranes are connected at sites where nuclear pore complexes (NPCs) are inserted, which direct the regulated transport of macromolecules in and out of the nucleus. The outer nuclear membrane contains integral proteins that connect it to the cytoskeleton, and in the luminal space these connect to the luminal parts of inner nuclear membrane proteins that in turn connect to the nucleoskeleton and chromatin. The primary structural support to the nucleus comes from the specific lamin nucleoskeleton that underlies the inner nuclear membrane and should be considered distinct from the nuclear matrix that supports chromatin inside the nucleus. Over the past decade or so it has become apparent that cancer cells have reduced stiffness and are strongly influenced by their biomechanical environment (reviewed in [61]). We now know that the nucleoskeleton is interconnected with the cytoskeleton. Thus, these biophysical/structural properties could also be involved in signaling to the nucleus through mechanotransduction, which could be very important in the unique microenvironment of a tumor that is very distinct from that of the surrounding normal tissue. It is also possible that an altered, less rigid, nuclear envelope could confer a significant advantage to metastasizing cells so that they can more easily migrate and invade surrounding tissue. The nucleus is the largest and most rigid of subcellular organelles, so a smaller or a less rigid nucleus would allow cells to squeeze through constrictions smaller than the diameter of their nucleus such as

between adjacent cells to escape from the vasculature endothelium or the epithelium surrounding a tissue. Disruption of nucleoskeletal–cytoskeletal connections has profound effects on nuclear positioning, nuclear migration, and cell migration [62, 63]. An advantage of increased nuclear size could be to provide a greater surface area for sequestration of regulatory factors. The lamins and several NETs have been shown to sequester proteins such as the tumor-suppressor retinoblastoma protein [64] and transcriptional regulators involved in tissue differentiation (e.g., Smads [65, 66]). Thus in theory a larger nucleus could sequester more of the tumor-suppressor or other transcription factors important for both cell cycle regulation and differentiation state of a cell.

From Microscopy to Biochemistry

The question "what is different in the nuclear envelope between a normal and a cancer cell?" was addressed initially by means of microscopy observations, but what is different between the nuclear envelopes of cancer and normal cells at a biochemical level?

Professor Ilya B. Zbarsky began to address this question in his laboratory by electrophoretic analysis of the proteins fractionated and extracted in different ways from crude nuclear preparations. In 1964 Zbarsky and co-workers identified a number of differences between the electrophoretic patterns obtained with normal and cancer cells [67]. Over the following decades his laboratory improved extraction procedures, using various nonionic detergents and nucleases to aid the extraction of proteins tightly bound to the nuclear membrane. In the meantime other laboratories specifically studying the nuclear envelope, particularly that of Nobel Laureate Günter Blobel at the Rockefeller University, developed procedures to specifically isolate nuclear envelopes [68]. It had been observed that there was a thick protein layer resistant to most chemical extractions used in biology that underlay the nuclear envelope and had been referred to as the fibrous layer or the nuclear lamina. From these studies with isolated nuclear envelopes they found that the most abundant proteins by far, almost certainly those of this lamina layer, were three polypeptides of around 65–70 kDa that were named lamins and corresponded to lamin A, lamin B1, and lamin B2 [68]. This enabled the Zbarsky laboratory in 1984 to identify lamins as the most prominent bands changing when comparing electrophoretic profiles of rat hepatoma against quiescent and regenerating normal liver cells. Furthermore, they found that proliferating cells showed an increase in lamin B and reduction of lamins A/C compared to non-proliferating cells [69].

Despite the biochemical identification of lamins in the mid-1970s, they were not known to be relatives of cytoskeletal proteins until a decade later. In 1984 Bob Goldman's laboratory isolated lamins from cultured cells and characterized them as keratin-like proteins, but did not himself realize that they were from the protein polymer underlying the inner nuclear membrane [70]. Finally in 1986 Frank McKeon, Marc Kirschner, and Daniel Caput [71] and Daniel Fisher, Nilabh Chaudhary, and Gunter Blobel [72] separately identified the lamins as intermediate

filament proteins. As such, the lamins have short N-terminal head domains (~33 amino acids) followed by a long rod domain (~350 amino acids) that homodimerizes to form four separate coiled coils separated by linkers for a linear length of ~52 nm followed by a large globular and variable C-terminal domain. The homodimers assemble into strands by head-to-tail interactions, and these strands then layer in an antiparallel fashion until there are 32 molecules in cross section to generate 10 nm wide filaments [73]. This assembly gives the lamins and other intermediate filaments unique properties compared to the other cytoskeletal proteins. Microtubules and actin filaments are built like stacked cinder blocks in a wall, whereas intermediate filaments are more like the entwined fibers of a rope, yet they are more tensile as the fibers can potentially move relative to one another—thus, it is not surprising that intermediate filaments are the primary components of spider's webs. Accordingly, actin filaments and microtubules will break under compression or stretching forces that leave intermediate filaments undamaged [74]. These characteristics are more important as the lamins are the only one of the three major cytoskeletal proteins giving structure to the nuclear envelope. However, even among the different lamin subtypes there are large differences in their contributions to mechanical stability. Lamin A was found to exhibit stronger binding in assembly assays compared to lamin B1, and lamin B2 was much weaker than both [75]. Correspondingly, lamin A has been found to be the most critical for mechanical stability [76]. Thus, though it provides the primary structural support for the nucleus, the nuclear envelope can nonetheless bend considerably in a migrating cell invading tissues and more so if lamin A is absent. This observation is more prescient in light of the fact that the most common observation with lamin levels in tumors is that lamin A is reduced, linking lamin abnormalities to the morphometric parameters used by cytologists.

While lamins initially received a great deal of attention, there are many other proteins in the nuclear envelope. The NPCs are large structures of >60 MDa in mammals containing around 30 different proteins in multiple copies (reviewed in [77]), and an average mammalian nucleus contains 2,000–3,000 NPCs. In addition to the NPCs, both outer and inner nuclear membranes contain a host of integral transmembrane proteins called NETs (for nuclear envelope transmembrane proteins). Just a decade ago, only a handful of NETs were known; however, in 2003, 67 novel NETs were identified in the laboratory of Larry Gerace by Eric Schirmer and colleagues [78]. A large proportion of the NETs were largely uncharacterized proteins of unknown function, with many of them exhibiting a marked tissue specificity in their expression. Today, close to 1,000 NETs have been identified [79–81], and a recent study comparing the nuclear envelope proteome of liver, muscle, and white blood cells showed that up to 60 % of the NETs may be preferentially expressed in a subset of tissues [81].

The tissue specificity of many NETs may contribute to the tissue-specific pathologies that occur with a set of nuclear envelope-linked diseases termed laminopathies. Many of these disorders manifest in a restricted number of tissues. For example, defects in the NETs emerin and the nesprins SYNE1 and SYNE2 as well as in lamins may result in Emery–Dreifuss muscular dystrophy. Intriguingly, different mutations in the *LMNA* gene (which encodes lamins A and C) can result in a variety of completely distinct diseases, each with different tissue-specific pathologies that can affect

heart (dilated cardiomyopathy), motor and sensory nerves (Charcot–Marie–Tooth disease), skeletal muscle (Emery–Dreifuss muscular dystrophy), fat (familial partial lipodystrophy), or skin (restrictive dermopathy). Lamin A mutations can also be associated with various forms of premature ageing, such as Werner's syndrome and Hutchinson–Gilford Progeria syndrome. How can mutations in a single ubiquitously expressed protein give rise to disease in some tissues and not others? The simplest answer would be through interaction with other factors that are tissue specific, a role for which many of these newly identified NETs stand out as good candidates.

These tissue-specific NETs could also contribute to the tissue-specific nuclear characteristics of many tumor types. In addition to the unexpected degree of tissue specificity present in the nuclear envelope proteome, NETs and lamins are being found to have functions in a variety of cellular processes, many of which can be linked to tumorigenesis (Fig. 4). Proteins of the nuclear envelope participate in cell cycle regulation, mitosis, apoptosis, DNA repair, ageing, nuclear architecture, signaling, chromatin organization, gene expression regulation, and cell migration. All these various functions are critical for processes of tumorigenesis, tumor growth, and metastasis (reviewed in [82, 83]).

We have recently investigated the gene expression profiles of nuclear envelope proteins in a microarray of tumor and normal samples from nine tissues available at the BioGPS database [82]. The microarrays contained probes for lamins A, B1, and B2 and for 29 NETs that had been verified by our lab and others [78–80, 84–94]. Most of the genes showed small and/or inconsistent levels of misregulation between and within tissues, but other genes showed some general tendencies. For instance, *LMNB1*, *LMNB2*, and *NUP210* were generally upregulated, and *METTL7A*, *SYNE1*, and *SYNE2* were generally downregulated (Fig. 5a). These tendencies were not absolute. *LMNB1* and *LMNB2* were not upregulated in prostate tumors, and in kidney tumors only *LMNB2* was upregulated. Additionally, we observed that in most gastrointestinal tumors *METTL7A* was upregulated rather than downregulated (de las Heras and Schirmer, unpublished results). Different tissues express lamins with subtype ratios that are characteristic of each tissue [95]. This coupled with the marked tissue-restricted patterns of NET expression may account for the tissue variability in the lamin and NET misregulation observed between tumors and suggests that some of these expression patterns may be exploited for diagnostic purposes. Some NETs show a particular potential to be used as markers for particular tumor types, such as *LPCAT3/MBOAT5* among a few others. *LPCAT3* does not show significantly consistent misregulation in eight of the nine tumor types studied but appears to be strongly upregulated in all of the ovarian cancer samples studied (Fig. 5b). We have also observed that some NETs were only strongly misregulated in a subset of tumors of only one type of cancer, such as *SLC22A24*, *NCLN*, and *FAM105A*, which were all upregulated in a subset of breast tumors (de las Heras and Schirmer, unpublished results). These differences may additionally reflect differences in tumor subtype or grade, but the BioGPS data did not contain enough information about the tumor samples to explore this possibility.

One area of study that is already showing translational promise is the targeting of nuclear import/export of proteins and RNAs through the NPC. Nucleocytoplasmic

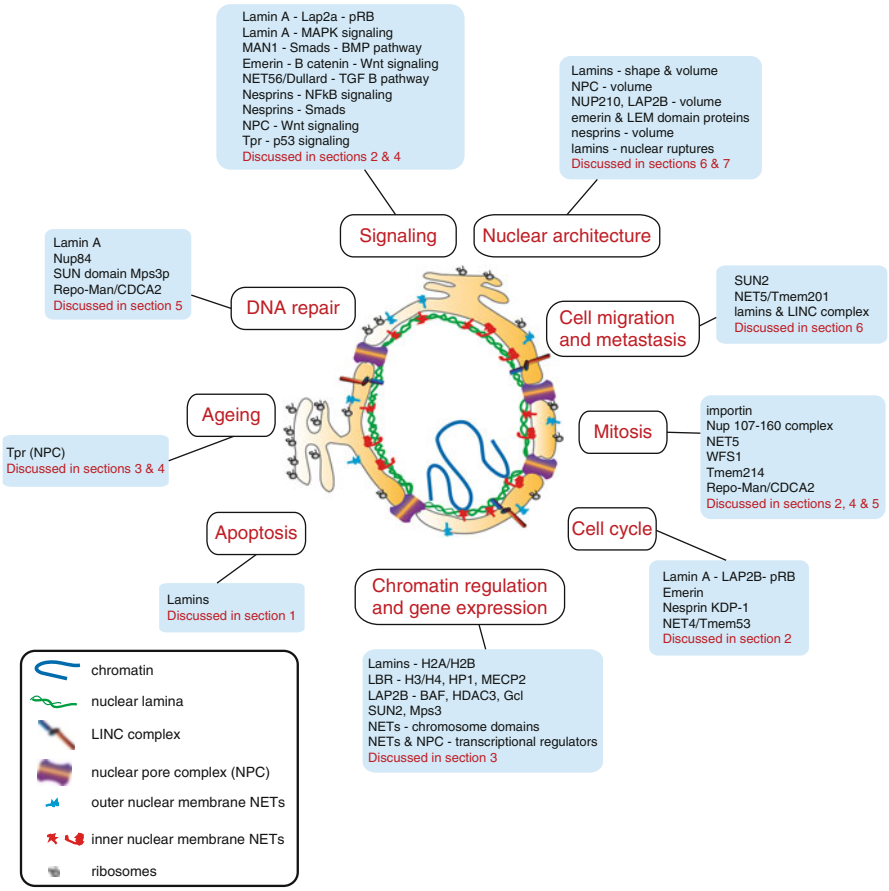

Fig. 4 Nuclear envelope functions with cancer links. The nuclear envelope comprises a double-membrane system studded with nuclear pore complexes and an underlying layer of intermediate filaments: the nuclear lamina. The nuclear envelope is connected to the cytoskeleton on the one side and chromatin on the other and acts as a powerful signaling node including pathways that are very relevant to cancer, such as Wnt and MAPK signaling. In addition, the nuclear envelope has been shown to play a role in many other functions that are relevant to cancer, such as control of nuclear architecture, cell migration, DNA repair, ageing, apoptosis, mitosis, and cell cycle regulation as well as genome organization and regulation of gene expression

transport is essential for cell growth and is often upregulated in tumors. Accordingly, the key nuclear export protein exportin 1 (XPO1/CRM1) has been found to be expressed at abnormally high levels in a number of cancers, and its inhibition promoted apoptosis and cell cycle arrest in cancer cells in vitro [96–99]. Clinical trials with initially promising results are currently under way using XPO1 inhibition in Philadelphia chromosome-positive (Ph+) leukemias, which are refractory to tyrosine kinase inhibitor therapy but appear to respond to an XPO1 inhibitor by triggering apoptosis of leukemic but not normal CD34+ progenitors [99].

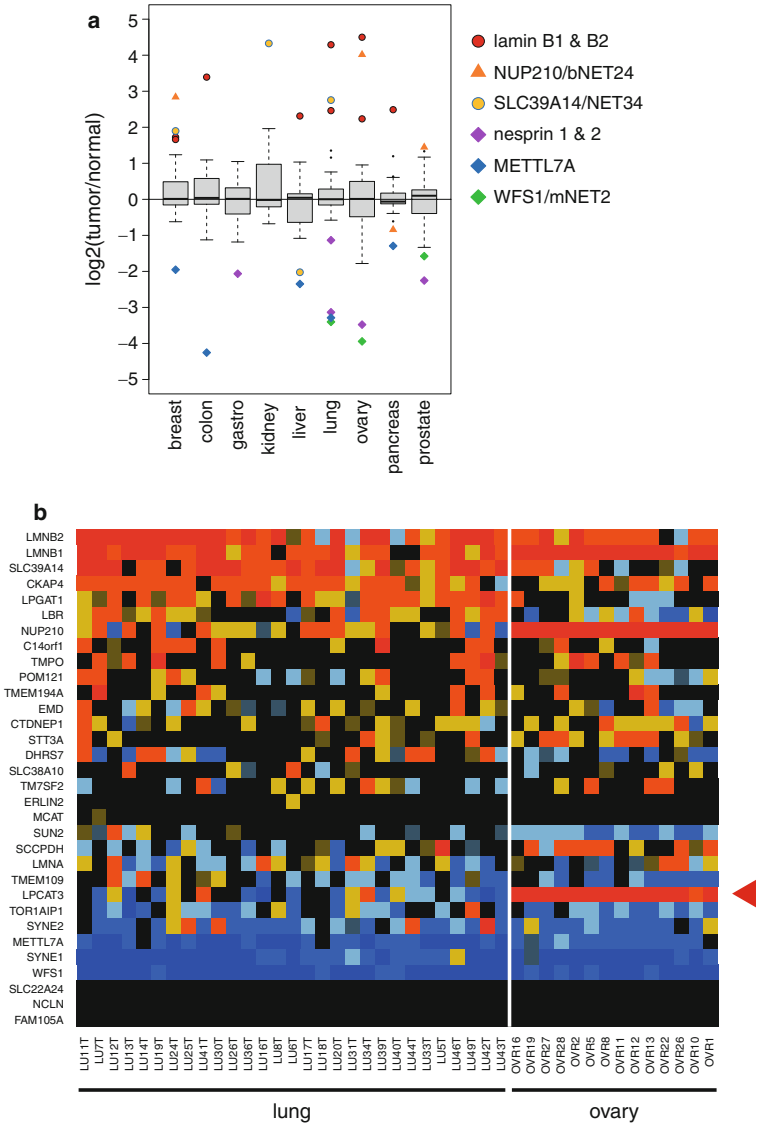

Fig. 5 Many nuclear envelope proteins are misregulated in tumors. (**a**) *Boxplot* showing the distribution of log2(tumor/normal) microarray signals for 29 nuclear envelope genes in nine tissues. The majority of the genes do not show a clear general misregulation in most tumors, but the genes that are most strongly misregulated are generally the same. Lamins B1 and B2 (*LMNB1* and *LMNB2*) and the nucleoporin *NUP210* are usually upregulated in tumors, while the protein methyltransferase *METTL7A* and nesprins *SYNE1* and *SYNE2* are almost always downregulated. However, some NETs, such as *WFS1*, are strongly downregulated in some tumors but not others, while *SLC39A14/NET34* is strongly upregulated in lung, kidney, and breast tumors and downregulated in liver cancer. (**b**) Heatmap illustrating the expression of 29 nuclear envelope genes in individual lung and ovary cancer patients, compared to their normal counterparts. A gradient of *reds* and *blues* indicate relative levels of up- and downregulation, respectively. The overall gene expression pattern is reasonably similar in lung and ovary patients; however, the tissue-specific NET *LPCAT3* (*red arrowhead*), which is normally expressed in the majority of normal tissues but not in ovary, is strongly upregulated in all ovarian tumors and downregulated in most lung tumors. Reproduced with permission from [82]

Concluding Remark

The more we learn about the nuclear envelope and its component proteins, the more it becomes apparent that the nuclear envelope, rather than representing an inert barrier between the cytoplasm and the nucleus, is at the center of many central cellular functions and processes, many of which have direct relevance to cancer biology. Over the past few years, the nuclear envelope has been shown to contain hundreds of NETs that are poorly characterized and of unknown function, many of which are altered in expression in various cancers. Many NETs showed altered expression patterns in cancer that suggest correlations with tissue and tumor grade. Together with the many clear links between lamins and NPC proteins and various cancers, this indicates that the nuclear envelope represents a novel, largely untapped, and potentially huge source for diagnostic and prognostic markers as well as for therapeutic intervention.

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