Distribution of Mutant Cells in Human Skin:  
Exploration of the Fetal-Juvenile Mutability Hypothesis

by

Leslie E. Kao

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Signature redacted
Department of Biological Engineering  
December 12, 2008

Signature redacted

Certified by:  
William G. Thilly  
Professor of Biological Engineering  
Thesis Supervisor

Signature redacted

Accepted by:  
Alan J. Grodzinsky  
Professor of Electrical, Mechanical, and Biological Engineering  
Chair, Department Committee on Graduate Students
ABSTRACT

The multiple "hits" carcinogenesis models are extensions of the cancer incidence theory developed by researchers from Nordling (1953), Armitage-Doll (1954 and 1957), Knudson (1971), Mooalgavkar and Verzon (1979), to Mooalgavkar and Knudson (1981), among others. These studies relate to the evolutionary process of normal tissue cells in an individual's organ from a normal stage to an initiated pre-neoplastic stage, and finally promoted to a neoplastic stage, resulting in tumorigenesis. The most significant impact of this type of research is to gain insight into the complex process of cancer development in humans.

In the case of skin cancer, epidemiological and molecular data clearly indicate that sunlight is a carcinogen, the primary cause of skin cancer, in which forms of point mutation are associated with ultraviolet radiation. Furthermore, sunlight is attributed both as a tumor initiator and a tumor promoter by favoring the clonal expansion of p53 mutated cells.

By utilizing studies of the multiple genetic hit model of oncomutation and inference that preneoplasia appears to be a clonal continuation of juvenile growth in adult tissues from which one stem cell creates an embryonic organ with lethal consequences, this thesis is devoted to the analysis of the process of skin cancer development and distribution of mutant cells in human skin, as well as the calculation of and inferences based on Gostjeva and Thilly's hypothesis on mutational clone frequency is restricted to the fetal-juvenile period.
ACKNOWLEDGEMENTS

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Finally, to my parents, Drs. George and Cassandra Kao, for their unconditional love and support.
LIST OF ABBREVIATIONS

AK  actinic keratosis
BCC  basal cell carcinoma
Cdk  Cyclin dependent kinase
CDCE  constant denaturant capillary electrophoresis
CPD  cyclobutane pyrimidine dimmer
CS  Cockayne syndrome
CSD  chronic sun damage
dNTP  denoxynucleotide triphosphate
CIS  cancer in situ
EPU  epidermal proliferative unit
IS  Internal standard
KA  keratoacanthoma
KC  keratinocytes
LOH  loss of heterozygosity
LOI  Loss of imprinting
MAMA  mismatch amplification mutation assay
MF(s)  Mutant fraction(s)
NBCCS  nevoid basal cell carcinoma syndrome
NER  nucleotide excision repair
NMSC  non-melanoma skin cancer
PCR  Polymerase Chain Reaction
PTCH  patched
SCC  squamous cell carcinoma
SHH  sonic hedgehog
SPR  sun protecting factor
SSCP  single strand conformation polymorphism
TCR  transcription-coupled repair
TTD  trichothiodystrophy
TUNEL  terminal deoxynucleotidyltransferase-mediated dNTP nick end-labeling
UV  ultraviolet
UVA  ultraviolet-A
UVB  ultraviolet-B
UVC  ultraviolet-C
UVR  ultraviolet radiation
UVS  ultraviolet sensitivity
WT  Wild-type
XP  xeroderma pigmentosum
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Chapter 1

Introduction

1.1. Purpose and Rationale of the Study

The reasons for and causes of cancer have flummoxed scientists for hundreds of years. The current theory is that of a multistage genetic hit model of cancer that predicts that normal individuals have stable populations of pre-cancerous mutant cells awaiting further genetic hits. Based upon previous observations and calculations, the team of Gostjeva and Thilly expanded upon this multistage model in their fetal-juvenile stem cell initiation hypothesis by identifying the at risk cells and a time frame in which the initial mutations would occur (1-2).

Observation of historical and present day age-specific colorectal rates, and direct measurements of genetic changes in human tissue as a function of age led Thilly and Herrero-Jimenez et al. to hypothesize that initiated stem cells grow at near juvenile rates to form a preneoplastic colony [3-4]. Further initiation would occur within this colony from which would emerge a neoplastic stem cell with near fetal growth rates to form a lethal tumor. Specifically, in the case of colon cancer, the calculated growth of the preneoplasia was equal to the growth rate of the juvenile colon, suggesting that tumor initiation blocked the developmental step that transformed a juvenile stem cell into an adult maintenance stem cell. Tumor promotion could be achieved by transformation of an initiated stem cell
within the slow growing precancerous colonic polyps into a fetal stem cell with a rapid net growth and the capability to differentiate [1]. While the multistage model of cancer formation had been uncovered, the question of identifying and visualizing the specific stem cells at risk still remained.

Through the reanalysis of fetal and adult colonic tissue with a novel histological preparation method, Gostjeva discovered that fetal and neoplastic tissues share a set of cells with unique non-spherical nuclear morphotypes which appear to cooperate in creating the elements of the fetal organ, preneoplastic and neoplastic lesions. Most tellingly, examination of fetal tissue showed the presence of tubular syncytia containing opened-mouthed, bell-shaped nuclei that underwent amitotic symmetric and asymmetric nuclear fissions, the later of which gives rise to seven other nuclear morphotypes. These other nuclear morphotypes subsequently divide extra-syncytially by mitoses to form clonal populations of cells with identical nuclear morphotypes in embryos, adenomas, adenocarcinomas and metastases. Bell-shaped nuclei have been found in, and thus appear to be responsible for both net growth and differentiation in the embryonic gut, adenomas and adenocarcinomas, and fulfill the requirements for post-embryonic stem cells in colon organogenesis and carcinogenesis [1]. Gostjeva and Thilly inferred that these bell-shaped nuclei represent the “metakaryotic” stem cells that drive net growth and differentiation in fetal, neonatal and juvenile development and in colonic adenomas, adenocarcinomas and metastases [2].

Further support for the fetal-juvenile stem cell initiation hypothesis came in the form of measurements taken from the lung by Sudo et al. in which it was found that
smoking and aging have no effect on the measured point mutant fractions nor on their numerical distributions in the adult lung epithelium. This lack of increase in point mutation fractions lead to the conclusion that the point mutations must have occurred before maturity. These mutant fractions are constant with age in adults because mutations occur much less frequently in adult maintenance stem cells than in pre-adult stem cells involved in fetal and juvenile growth. Furthermore, Sudo et al. proposed that the measured mutations arose during an exponential increase in stem cell number during human organ growth and development, possibly within Gostjeva’s metakaryotic cells [5].

Concurrent with the research done on the genetic factors leading to cancer, much study has been done examining the possible effect of environmental factors on the progression of carcinomas. Epidemiological analyses of age-specific cancer rates show large historical increases. Since inherited genetic conditions could not make an appreciable change in only a few generations, environmental factors had to be the catalyst. Supported by the fact that the large increases in age-specific mortality rates for several forms of cancer occur concurrently with the population shifts from the agrarian to urban, the abundance of potential human mutagens in our daily environment and the resulting products of chemical reactions between environmental chemicals and DNA or proteins in human tissues can be pointed to as the catalyst for the historical increase in cancer rates [6].

This thesis will rely upon skin cancer to test the validity of both the genetic and environmental theories.
1.2. Aspects of Skin Cancer Study

By reanalyzing data from Brash, Pontén and Jonason *et al.*'s research and findings [7], and applying current theoretical hypotheses, this thesis is designed to investigate the mechanism and development of skin cancer [7]. Two aspects will be emphasized:

First. Sunlight is an environmental carcinogen that acts as both a tumor inducer and promoter. Skin cancer is the only cancer that provides a link between skin cell mutations and sunlight, in which forms of point mutations are associated with ultraviolet radiation (UVR) [6]. Brash *et al.* stated that sunlight is a carcinogen – an “initiator” which produces mutations and leaves its molecular footprint, or “signature,” in the DNA C→T transitions at dipyrimidine sites predominate, including CC→TT double-base mutations [10].

In addition, in their study of frequent clones of p53-mutated keratinocytes in normal human skin, Jonason *et al.* stated that normal human skin contains multiple colonies of keratinocytes that stain for aberrant p53 protein [8]. By observing that higher frequency and larger mutant patch sizes occur in sun-exposed skin, they concluded that sunlight acted as a tumor inducer as well as a tumor promoter, providing selective growth advantage to both endogenous and exogenous mutants [8-9].

Second. Distribution of mutant cells in human skin and stem cell mutation limited to the fetal-juvenile period hypothesis:

Their study also observed that p53 patch frequency is independent of age based upon the finding that substantial mutagenesis seems to occur during childhood and much
less during adulthood, if any [8]. Similar epidemiological evidence relating to sunlight exposure before age 18, as noted by Marks, Kricker, English, Leffell, Ziegler, Pontén, Ren, and Brash et al. [10-24] closely parallels this thesis’ second focus to apply the Gostjeva-Thilly hypothesis in which skin cancer in adults occurs at constant rates in pre-adult stem cells involved in fetal and juvenile growth as implied by the fetal-juvenile mutator stem cell hypothesis [1-2].

1.3. Scopes of Skin Cancer Study

Accordingly, emphasis for this thesis is twofold: The first part focuses on human skin, skin precursors, skin cancer types, and offers a review of relevant research and scholarship. Subsequently, by utilizing studies of the multiple genetic hit model of oncomutation in developing fetal juvenile tissues, the second part of this thesis is devoted to the analysis of the process of skin cancer development, p53, distribution of mutant cells in human skin, as well as the calculation of and inferences based on Gostjeva-Thilly’s fetal-juvenile hypothesis.
Chapter 2

On Skin Cancer: Background Review

2.1 On Human Skin

Consisting of approximately one sixth of the human body’s total weight, the skin is the largest human organ. In its physiological anatomy, the skin consists of several layers: the epidermis is the outermost layer with an inner dermis layer in conjunction with subcutaneous tissues. See Figure 1:

![Figure 1: Anatomy of the Skin](http://www.cancer.gov/cancertopics/wyntk/skin/page2)
Up to 90% of the cells in the epidermis are keratinocytes (KCs), which function as a barrier preventing the invasion of harmful substances and minimizing water loss from the body through the epidermis. The other portion of the epidermal cells are melanocytes, called stratum basale, which produce melanin, the protein that adds pigment to skin keratinocytes and thus protects the human body from ultraviolet rays (UVR). In general, the skin darkening process due to sunlight exposure results from the processed movement of existing melanin into keratinocytes, along with the increase of melanin by the melanocyte [7].

As indicated in Figure 1, the dermis, or the “true skin,” is located under the epidermis. The dermis is composed of elastic materials, water, and collagen. Embedded in this layer are the blood vessels, lymph vessels, and glands. While certain glands produce sweat to cool the body, other glands make sebum to keep the skin from drying out. Both sweat and sebum reach the skin surface through small openings called pores.

The epidermis is comprised of five distinct layers of stratified keratinized epithelium that represent a progressive maturation and differentiation of the keratinocytes. The deepest layer, the *stratum germinativum* or *stratum basale*, is a single layer of columnar basal cells that undergo mitosis to produce new keratinocytes that move to the skin surface, replacing cells lost during normal skin shedding. The basal cells remain stationary in the *stratum germinativum*.

With an observed pathology curiously similar to Gostjeva’s bell-shaped nuclei, columnar cells possess ovoid or “indented” nuclei with one or two nucleoli embedded in
euchromatin surrounded by a thin rim of heterochromatin. Occasionally two or three rows of these cells may be present with the appearance of smaller oval daughter cells through mitosis. The mitotic index is approximately 2 cells for every 1000 cells.

The next deepest layer, the *stratum spinosum*, is formed from the progeny of the basal cell layer as they move outward. Two to four layers thick, the “pickle” cells of the stratum spinosum differentiate as they move outward. The *stratum granulosum* consists of granular cells that are the most differentiated cells of the living skin. While some cells in this layer loose cytoplasm and DNA structure, others continue to synthesize keratin. Mostly confined to the palms of the hand and the soles of the feet, the *stratum lucidum* lies adjacent to the stratum granulosum and appears as a thin, transparent layer that retains some function of living cells from deeper layers but otherwise resembles cells of the *stratum corneum*. This top or surface layer is composed of dead keratinized cells ranging from 15 to 25 to 100 layers thick in some regions.

The epidermis is maintained through the proliferation of keratinocytes, which retain the ability to self-renew throughout one’s life time and produce daughter cells, which undergo terminal differentiation and death [7, 25-26]. More explicitly, the epidermis is comprised of an epidermal proliferative turnover unit in which a skin cell residing in the basal layer populates a defined region of overlying skin [27]. Human skin is a fine mosaic of “tiles” or clusters of clonally derived cells consisting of around 20 to 350 basal keratinocytes [28].

The significance of keratinocytes lies in its continuous proliferation, differentiation, and apoptosis via its stem cells, transient-amplifying cells, and committed cells [29]. In
comparison, columnar basal cells represent around 10% of the keratinocyte cell population, generating daughter cells from mitosis which are either stem cells or transient-amplifying cells. Transient-amplified cells represent around 50% of the basal cell population, producing daughter cells that lead to stages of terminal differentiation and death [29].

2.2. Epidemiology and Skin Cancer

Skin cancers are categorized according to their cancerous cell of origin: In melanoma skin cancer (MSC) -- melanoma from melanocytes or melanocyte precursors, and nonmelanoma skin cancer (NMSC) -- around 80% of the tumors are basal cell carcinomas and 20% are squamous cell carcinomas. The two common types of cancer under the nonmelanoma skin cancer category are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [30].

According to the National Cancer Institute, skin cancer, the broad category for cancers arising from the different cells that make up the skin, is the most commonly diagnosed cancer type in the United States. An estimated 59,940 new cases (33,910 men and 26,030 women) were diagnosed with melanoma of the skin in 2007 – 8,110 of which are predicted to die of this type of cancer. In 2007 in the category of nonmelanoma skin cancer (NMSC), more than 1 million new American cases were diagnosed (www.cancer.gov/cancertopics/wyntk/skin).

Broken down by race, Figure 2 below includes death rates due to the melanoma skin cancer between 2000 and 2004. The age-adjusted death rate was 2.6 per 100,000 men and women per year -- white males and females constitute the highest death rate with 4.3
deaths per 100,000 white men, and 2.0 deaths per 100,000 white women. Males and females of Asian and Pacific Islander decent constitute the lowest death rate at 0.4 per 100,000 for Asian/Pacific Islander males, and 0.3 per 100,000 for Asian/Pacific Islander women:

Figure 2:

<table>
<thead>
<tr>
<th>Race/Ethnicity</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Races</td>
<td>3.9 per 100,000 men</td>
<td>1.7 per 100,000 women</td>
</tr>
<tr>
<td>White</td>
<td>4.3 per 100,000 men</td>
<td>2.0 per 100,000 women</td>
</tr>
<tr>
<td>Black</td>
<td>0.5 per 100,000 men</td>
<td>0.4 per 100,000 women</td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>0.4 per 100,000 men</td>
<td>0.3 per 100,000 women</td>
</tr>
<tr>
<td>American Indian/Alaska Native</td>
<td>1.3 per 100,000 men</td>
<td>0.7 per 100,000 women</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0.9 per 100,000 men</td>
<td>0.6 per 100,000 women</td>
</tr>
</tbody>
</table>


Figure 3 below offers mortality rates in the United States due for melanoma of the skin between 1988 and 1992 (from National Cancer Institute, SEER Program, *ibid.)*:  

19
MELANOMA OF THE SKIN

United States MORTALITY Rates by Age at Death, 1988-1992

AGE 30-54

AGE 55-69

AGE 70+

NOTE: Rates are "average annual" per 100,000 population, age-adjusted to 1970 U.S. standard. N/A = data unavailable; *= fewer than 25 deaths.
The following skin cancer age-year-specific and birth-year-specific mortality data in Figure 4-A & B and Figure 5-A & B collected and organized by Herrero-Jimenez of the Thilly group, are available at http://epidemiology.mit.edu [3-4]. These are quantitative analyses of public health data for skin cancer in the US were maintained by the US Census Bureau (1870-1935) and later by the US Public Health Service (1936-1997):

Figure 4-A: Skin Cancer

![Skin Cancer (EAM)](image)

Figure 4-A: This is skin cancer age-year-specific and birth-year-specific mortality rates From http://wgt5.mit.edu. EAM stands for European-American males.
Figure 4-B: Skin Cancer

Figure 4-B: This is skin cancer age-year-specific and birth-year-specific mortality rates. From http://wgt5.mit.edu. EAF stands for European-American females.
Figure 5-A: Skin Cancer

Figure 5-B: Skin Cancer

Figure 5-B: Skin cancer age-year-specific and birth-year-specific mortality rates
2.3. Pathology/Histopathology and Skin Cancer

2.3.1 Melanoma Skin Cancer (MSC)

Melanoma skin cancer (MSC), also known as malignant melanoma, is a malignancy which originates from melanocytes, the pigment-producing cells in the skin [31]. Early melanomas are seen clinically as flat, pigmented macules that mostly follow the ABCD rule - asymmetry, border irregularity, color variegation and lesional diameter > 6 mm [32-33]. Histopathologically, neoplastic melanocytes of melanoma in situ are located within the epidermis, where focal proliferation in the basal layer occurs in early stages [33]. Due to the fact that the nuclei of these cells at this initial stage may appear normal, accurate histopathological diagnosis may be impossible. This type of diagnosis only becomes possible after some time has passed, during which solitary melanocytes extend horizontally along the basal layer and develop atypical nuclei. These atypical melanocytes ascend to form poorly demarcated nests of variable sizes and shapes [33].

2.3.2 Nonmelanoma Skin Cancer (NMSC)

Nonmelanoma skin cancer is most common among white populations (see Figure 2). Around 80% of the tumors are basal cell carcinomas (BCCs), the remaining 20% can be categorized as squamous cell carcinoma (SCC) [50]. Putative precursor lesions (e.g. actinic keratoses) to SCCs are very common. As shown in Figure 2, NMSC is the most common human cancer in predominantly Caucasian populations of certain areas of the world - such as the high percentage presented with NMSC each year in the United States and Australia [11, 34]. Marks’ 1989 and 1990 reports state that over half the population
above the age of 40 has NMSCs or cognate lesions in Australia due to high ambient ultraviolet radiation and a predominantly white population [11-12].

2.3.2.1 Basal Cell Carcinoma (BCC)

A typical BCC arises from the basal cells that form a border between the dermis and the epidermis. BCCs occur as small, shiny pink or pearly lumps with an ulcerated center and are most commonly found in sun-exposed skin, such as one’s face, and less commonly on the upper trunk or non-exposed place. From a histological point of view, the tumors display downgrowths of keratinocytes from the epidermis lined by palisades of cells resembling basal cells [30]. Since BCCs do not metastasize, they are almost always harmless and can be cured at any stage if excised completely via surgical therapy or radiotherapy, which explains the low death-rate for BCCs [30].

2.3.2.2 Squamous Cell Carcinoma (SCC)

Squamous cell carcinomas (SCCs) can be defined as invasive tumors with the ability to metastasize [30]. Originating in sun-exposed skin, SCCs grow quicker than BCCs, and create more irregular keratotic lesion with ulceration. In SCCs, metastasis rates are between 0.1% and 4%, and, the overall mortality is low [30]. Both cutaneous malignant melanoma and squamous cell carcinomas arise from the squamous cells in the epidermis and have an initial progression stage in which proliferation of the neoplastic cells are mostly confined to the epidermis [33].
Early cutaneous squamous cell carcinoma (SCC) occurs in several different ways involving multiple lesions that are clinically characterized according to the carcinogenic stimuli which cause the epidermal keratinocyte transformation, including solar keratosis, thermal keratosis, arsenic keratosis, radiation keratosis Bowen’s disease, bowenoid papulosis, and leukoplakia [33]. These lesions are considered precursors to cutaneous SCC because they are: (i) biologically benign with only a few progressing to invasive SCC; (ii) histopathologically atypical — made up of large, pleomorphic nuclei; (iii) cytologically indistinguishable from the atypical keratinocytes of invasive SCC; and (iv) clinically as well as histopathologically, no well-defined boundary exists between precursor lesions and lesions of invasive SCC. If left untreated, precursor lesions have the potential to become malignant, metastasizing deeper tissues [33].

Considered the most commonly observed form of early cutaneous SCC, solar keratoses usually occur as multiple lesions, <1 mm in diameter, and covered by adherent scales. Lesions are more often found in the sun-exposed skin of fair-skinned elderly persons with the atypical solar keratinocytes in the basal layer of the epidermis. Later proliferation occurs in the mid- to upper epidermis in which the keratinocytes lose polarity and arrange themselves in a disorderly fashion [33].
Chapter 3

On Skin Cancer and Risk Factors: Literature Review

3.1. Environmental Causes and Ultraviolet Radiation (UVR)

Epidemiological and molecular data clearly indicate that sunlight is a carcinogen to which everyone is exposed [10, 13-14]. Sun-exposure is also attributed as the primary risk factor and main environmental cause of skin cancer [10, 13-17], which causes DNA damage, sunburn, mutation-associated inactivation of the p53 tumor suppressor gene and clonal expansion [10, 13, 17, 19, 35].

3.1.1 Ultraviolet Radiation as a Carcinogen

The ultraviolet radiation (UVR) contains three spectra that are distinguished by their respective wavelengths: UVA containing wavelengths from 320 nm to 400 nm; UVB, from 290 nm to 320 nm; and UVC, from 100 nm to 290 nm [10]. While UVC is filtered out by the ozone layer, both UVA and UVB can reach the surface of the earth. UVA, which constitute 95% of surface UVR rays, is absorbed by the dermis and cause the skin photoaging of the skin, while UVB is absorbed by the epidermis and has been demonstrated to cause DNA damage [10, 18, 29].
Ultraviolet light produces mutations and leaves marks in the DNA. UVR induced mutations are C → T transitions, occurring at dipyrimidine sites, and include CC → TT double-base mutations (10).

3.1.2 Sunlight Exposure Risk Factors and Epidemiologic Features

Six categories based on higher frequencies that indicate sunlight as the main risk factor for human skin cancer have been identified by English et al. [15]. These six are: (1) people living in areas of high ambient solar irradiance; (2) people with sun-sensitivity; (3) people with high sun-exposure; (4) people who expose more body area to the sun; (5) people with benign sun-related skin conditions; and (6) people less damaged by more skin protection against the sun [15].

The epidemiology of skin cancers includes melanoma and nonmelanocytic skin cancers (squamous cell carcinoma [SCC], and basal cell carcinoma [BCC]), as well as melanoma of the eye [15]. English et al. divide these cancer risk factors into three distinct categories for further analytical studies: (1) Descriptive Category (including describing people’s ethnic origin, place of residence, migration, occupation, and anatomic site); (2) Analytic Category (including analyzing the sensitivity of people’s skin to sunlight, ambient sunlight at places of residence, exposure of the skin to sunlight [total exposure, occupational exposure, non-occupational exposure, and sunburn], other sun-related skin damage, and protection against sunlight); and (3) Molecular Effects Category (such as effects of DNA repair deficiency, and mutations in tumors characteristic of UV radiation)
The summarization of these epidemiologic features highlighting sunlight’s role in causing melanoma, BCC, and SCC cancers as reflected in Figure 6 below:

Figure 6:

<table>
<thead>
<tr>
<th>Epidemiologic Features</th>
<th>Melanoma</th>
<th>BCC</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
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<td>1. Descriptive Category:</td>
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<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Migration</td>
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<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Occupation</td>
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<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Anatomic site</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>2. Analytic Category:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity of skin to sunlight</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Ambient sunlight at residence places</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Exposure to sunlight:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total exposure</td>
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<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Occupational exposure</td>
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<td>yes</td>
</tr>
<tr>
<td>Non-occupational exposure</td>
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<tr>
<td>Sunburn</td>
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<td>yes</td>
<td>yes</td>
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<tr>
<td>Other sun-related skin damage</td>
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<td>yes</td>
</tr>
<tr>
<td>Protection against sunlight</td>
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<td>no</td>
<td>no</td>
</tr>
<tr>
<td>2. Molecular effects of ultraviolet radiation:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effects of DNA repair deficiency</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Mutations in tumors characteristic of UV radiation</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

Summary of D. R. English’s Epidemiologic Features of Skin Cancers: Melanoma, BCC, and SCC [15]
The above chart reveals that various details related to sunlight are indicated as potential causes of skin cancer. However, there are some differences among melanoma, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC). For instance, in the second category, both melanoma and basal cell carcinoma (BCC) are implicated more with non-occupational exposure than occupational exposure to sunlight. Squamous cell carcinoma (SCC), on the contrary, is related more to occupational and non-occupational exposure to sunlight [15].

3.2. Studies of Population Migrations

Various studies conducted by English, Leffell, Kricker, and Marks et al. on the migrations of white Australians have illustrated the distribution of epidemiological features [11-17, 20-21].

3.2.1 Ethnic Origin and Place of Residence

Early epidemiologic studies conducted as far back as 50 years ago, show that white Australians – who predominantly descended from the fair skinned, light haired populations of the British Isles – experience the highest rates of all types of skin cancer in the world. Their genetic relatives, living under cloudier conditions were more fortunate, as were the darker skinned Australian Aborigines with whom they shared an environment [16]. In the United States, the incidence of skin cancer is about 20-fold higher in Whites of European origin than in Blacks [15]. Melanomas and NMSCs are the densest in chronically sun-exposed sites (head and neck) and the least dense in rarely exposed sites (buttocks and
abdomen). As a result, fair skin and strong sun exposure have been identified as strong risk factors for skin cancer [15].

3.2.2 Migration Origin

In addition, studies that when people of European origin migrate from places of low incidence melanoma and low ambient sunlight to places of high incidence and high ambient sunlight, subsequent rates of melanoma were higher than those of their home country. Kricker [20], Marks [21], and English [15] further noted that people who had migrated from the lower ambient sunlight of England before the age of 18 to Australia with its high ambient sunlight acquired the higher Australian incidence of NMSCs. However, if migration occurred after the age of 18, they retained the risk of their country of origin [33-34]. These findings suggest that Australian skin cancer patients receive a critically high dose of sunlight years before the appearance of tumors, which typically arise in patients, between the age of 50-70 [7, 16]. This aspect will be discussed further in the next two chapters.

3.3 Case Study of International Variations on Pigmentation and Malignant Melanoma Incidence Rates

There is a clear correlation between malignant melanoma incidence rates and racial composition, and the intensity of sunlight exposure in different geographic areas. Rates are low in races with the most skin pigmentation, such as blacks and Asians, and are high in whites. Among whites, as indicated by Marks et al., incidence rates are about four times higher in Australia and New Zealand than in England and Scotland [21]. According to the
SEER of the National Cancer Institute, among white populations in the United States, the risk for malignant melanoma is highest for fair-skinned people, while Asian, black, and Hispanic people are among the lowest groups. The differences in pigmentary characteristics between different populations are largely attributed as one of the risk factors that lead to malignant melanoma incidence rates, as indicated in Figure 7 below (http://www.cancer.gov/cancertopics/wyntk/skin):
Figure 7:

MELANOMA OF THE SKIN

SEER INCIDENCE Rates, 1988-1992

United States MORTALITY Rates, 1988-1992

NOTE: Rates are "average annual" per 100,000 population, age-adjusted to 1970 U.S. standard; N/A = information not available; * = rate not calculated when fewer than 25 cases.

National Cancer Institute 85 SEER Program
3.4. Risk Factor of Familial Disorders, PTCH and the Hedgehog Pathway

3.4.1 Gorlin Syndrome (NBCCS) and PTCH:

The most common familial disorder is the nevoid basal cell carcinoma syndrome (NBCCS, Gorlin syndrome), which roughly constitutes less than 1% of all cases of skin cancer [30]. In Gorlin syndrome, BCC is the most prevalent tumor which features an autosomal dominant disorder characterized by multiple BCCs with onset at an early age and with many forms of congenital malformations, such as keratocysts of the jaw. Brash et al. stated that patients with Gorlin syndrome usually inherit a point mutation and loss of the second allele in a single cell that causes either a tumor or the aforementioned congenital malformation [18]. The gene responsible for NBCCS was mapped by groups to 9q22-31 and identified as the patched gene, PTCH, which is a human homologue of the Drosophila gene patched (PTC) [18, 29-30, 32]. Brash et al. explained further that PTCH is mutated in about 90% of BCCs, and that inherited mutations in the gene cause Gorlin syndrome [18].

3.4.2 PTCH and Hedgehog Pathway

Brash and Pontén further explained that the sequencing of BCCs indicated that nearly all BCCs contain PTCH mutations. PTCH encodes for a large glycoprotein with 12 membrane-spanning domains and two large extra-cellular loops. Previous Drosophila studies stated that this glycoprotein is part of the hedgehog signaling pathway, which played an important part in early patterning determination and cell fate in which PTCH participates in a complex with a membrane protein, smo. Uncoupled, smoothened appeared
to be active, while the complex with patched inhibited smo activity through the binding of the secreted hedgehog signaling protein. Inhibition of smo induces transcription of smoothened downstream target genes. In its inactive form on which the hedgehog pathway was placed, patched would be the most visible. An inactivating mutation in patched would lead to an increase in its transcription, as evidenced in BCC tumors [7].

Gailani et al. showed that expression of PTCH was barely detectable in the epidermis, while readily detectible in tumors, suggesting upregulation of the gene only after both alleles of PTCH were mutated [36]. Brash and Pontén suggested that PTCH inactivation resulted in skin tumors through the resulting upregulation of other hedgehog target genes, specifically sonic hedgehog (SHH), the most common vertebrate homologue of hedgehog required for correct patterning of the neural tube and somites, and for anterior/posterior positioning of the limb bud. Transgenic mice with overexpression of SHH have been shown to develop many features of Gorlin syndrome [7].

Gailani et al. also reported that the loss of heterozygosity (LOH) data had been located in more than half of sporadic BCCs [37]. Finally, the PTCH mutations fit Knudson’s two-hit hypothesis that tumors in inherited cancer predisposition syndromes have mutations of the same gene as sporadic ones in the same tumors [29, 38-29].

3.5 Stem Cell and Hypothesis-related Studies

Coller et al. and Sudo et al.’s clustering of mutant mitochondrial DNA copies [40-41, 5] and stem cells [42], Gostjeva-Thilly’s stem cell and multiple human fetal organs [1-2], and Sudo et al.’s fetal-juvenile origin of nuclear point mutations and lung cancer [5], will support the Gostjeva-Thilllly hypothesis that cells with bell-shaped nuclei are
responsible for net growth and differentiation in the embryonic gut, adenomas, and adenocarcinomas, and be used to fulfill the requirements for post-embryonic stem cells in carcinogenesis [1-2]. Chapter 5 will devote to the analysis of the Gostjeva-Thilly hypothesis of the fetal-juvenile mutability hypothesis and its inference to age-independence in skin cancer.
Chapter 4

Multiple Genetic Hit Process
of
Skin Cancer Development

4.1 Multiple Genetic Hit Theory of Cancer Development

Carcinogenesis is caused by a multistage process, in which mutations play a pivotal role in cancer development [43]. The stepwise process usually starts with the accumulation of mutations that promote clonal selection of cells and eventually leads to a lethal metastatic tumor [3-4, 9, 38, 44-48].

4.2 Multistage Process of Skin Cancer Development

Since sunlight – the ultraviolet radiation (UVR) from the sun-exposure – is the most prevalent human carcinogen, skin cancer is considered an ideal case to study its multistage process leading to skin carcinogenesis. Brash et al. have stated that the stages starting with genetic events, which are preceded by molecular events that create the mutation. Cellular events follow and allow a cell to acquire another mutation to progress to a tumor, as seen in the following Figure 8, while illustrate the genetic and cellular events in the early development of skin cancer [48]:

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Based on Brash et al.'s "Genetic and cellular events in the early development of skin cancer" [48].
Summarizations of Figure 8 are as follows [48]:

1. Sunlight and its ultraviolet radiation acts as the initiator.
2. Sunlight creates first cyclobutane dimmers and photoproducts in DNA [18], leading to C→T mutations at dipyrimidine sites [49] in the p53 and PTCH tumor suppressor genes [10, 17, 23, 50].
3. The expansion of a single mutant cell to a clone of 1,000 cells allows one of the 1,000 cells to begin another pass through the mutation loop.
4. One phenotype of losing p53 is the loss of UV-induced apoptosis of DNA-damaged cells resulting in the accumulation of mutant cells [17].
5. Subsequently, the clonal expansion of the mutant cell leads to the acquisition of a second mutation – which makes the accumulation of multiple mutations possible [48].
6. Finally, as a promoter, sunlight will continually creates mutant keratinocytes, and drive their clonal expansion [48].

In reviewing the chart above, “clonal expansion” is the key step for the multiple genetic hit mechanism in skin cancer development. In the following, the topic of clonal expansion relating to multiple genetic hit carcinogenesis will be analyzed further.

4.3 Sunlight, Clonal Expansion, and p53

4.3.1 On p53 Gene, p53 Mutations, Frequency, Selection, and Clonal Origin
SCC: The p53 is the most studied tumor suppressor gene. It is involved in both BCC and SCC [46]. Brash and Ziegler et al. also observed that over 90% of human SCCs have the p53 mutations induced by ultraviolet radiation [10, 50].

Furthermore, p53 mutations are present in carcinoma in situ [7]. The p53 protein is a transcription factor whose targets include genes that regulate cell cycle and cell death [7]. In addition, over 60% of dysplasias contain p53 mutations [7, 17]. According to Brash et al., the presence of p53 in dysplasias reveals that these precancers are clonal events rather than toxicity reactions. Different parts of the same dysplasia have identical mutations, confirming a clonal origin [7]. However patients with multiple dysplasias have different p53 mutations in each lesion suggesting that each dysplasia originated from a distinct mutational event [17]. Since each mutation changes the amino acid, it appears that the mutations have been selected as opposed to being an incidental side-effect of dysplasia [7].

BCC: p53 mutations are also present in nearly all BCCs, including small, presumably early lesions [26, 51]. In all cases, p53 mutations surface through the lesion suggesting that the mutations arise prior to the emergence of the dysplasia. To further characterize the onset of the mutation and determine whether dysplasia arising within a large p53 mutated clone of cells in skin damaged skin, Ziegler et al examined biopsy samples of normal skin surrounding the dysplasia for the same mutation as that of each dysplasia [17]. The frequency of such mutations are found from $\sim 10^{-4}$ in Brash et al., and from $10^{-3}$ to $10^{-2}$ in Gailani et al. study [8, 10, 36], suggesting that the p53 mutations arise at the same time as the clonal expansion of the tumor. Frequency of mutations is presented
to be higher in sun-exposed skin — for example, from $10^{-4}$ to $10^{-3}$ in Jonason et al study — than in non-sun-exposed skin [37, 8].

**Hotspots:** Mutations in SCC and BCC are clustered into hotspots with the amino acid substitutions spread evenly across the p53 gene suggesting that some p53 alleles can lead to an SCC, whereas others are more likely to regress [17]. Another possible explanation, coinciding with the findings of Thilly group, is that the regressed p53 mutations occurred in non-stem cells, leading to their ultimate turnover and disappearance from the skin tumor [5].

4.4. Sunlight, Clonal Expansion, Apoptosis, Stem Cell Compartment of the Epidermis

**The acquisition of a second mutation:** Studies about the sun-exposed skin of Caucasians contain thousands of p53-mutated clones, which are clinically invisible but can be recognized in skin immunostained with the p53 antibody. UV-related p53 mutations -- C to T or CC to TT transitions at dipyrimidine sites -- are confirmed in the p53 mutated clones by microdissection and sequencing [7-8, 10, 17, 33]. The p53 gene is critically involved in the regulation of cell cycle control and apoptosis. Mutations in the p53 gene could potentially abrogate p53 function, allowing for the accumulation of mutant cells due to loss of the UV-induced apoptosis of DNA-damaged cells. This leads to clonal expansion of p53-mutated keratinocytes, creating a larger target for the acquisition of a second mutation. The second mutation will lead to the progression of clinically recognizable solar keratosis.
4.4.1 UV Drives Clonal Expansion of p53-mutant Keratinocytes in Normal Skin

The results reported by Brash, Pontén, Ren, Jonason, and Ziegler et al. have shown that epidermal p53 clones are frequently detected in chronically sun-exposed sites [7-8, 22-24, 50, and 52]. Furthermore, the data presented by Brash et al. have shown that heavily sun-exposed skin would be more likely to sustain a mutation early in life, and that these early cells could grow into a clone even without further sunlight [48].

The study conducted by Jonason et al. revealed a much comprehensive report of the multiple genetic hit model of carcinogenesis which predicts that ordinary people have cancer-prone mutant cells dormant for further genetic hits [8], consistent with Marks, Kricker, English or Ziegler et al. claim that critical sunlight exposure is received before the age of 18. In addition, Jonason et al.'s article furnished an invaluable link between the time of initial sun-exposure and subsequent skin cancer years later, claiming that sunlight acts as both a tumor initiator and tumor promoter by favoring the clonal expansion of p53-mutated cells. In their study, both patch size (sun-exposure dependent) and frequency are independent of age [8].

4.4.2 Clonal Expansion involving UV-induced Physiology, not Mutation

In their study of the colonization of adjacent stem cell compartments by mutant keratinocytes, Brash, Jonason et al. provided detailed counts of clone sizes that indicate the quantized distribution of sizes of p53-mutant clones. They stated that the occurrence of quantized clone sizes reveals the fact that a rate-limiting step in clonal expansion is the ability to expand beyond the first stem cell compartment [48]. UVB exposure drives
clonal expansion by a non-mutational mechanism, allowing mutant stem cells to escape from their own stem cell compartment and colonize adjacent compartments – similar to J. Cairns’ safety mechanism proposed in 1975 [53].

4.4.3 Remarks on Clonal Expansion and UV-induced Apoptosis

The regular role for UVB-induced apoptosis is to delete DNA-damaged cells in unmutated stem cell compartments, while sparing death-resistant p53-mutant cells [17, 48]. Consequently, the effect of apoptosis on cancer development is stage-specific: apoptosis suppresses two stages of cancer development that require new mutations (i.e. initiation and malignant conversion). However, apoptosis can drive clonal expansion, for both existing p53-mutant cells and for papilloma precursors [48]. Brash et al. pointed out that the additional source of apoptotic selection pressure may come from UV-irradiated melanin [48].

The conclusive remarks for this chapter are that ultraviolet radiation is the carcinogen for human skin cancer, and clonal expansion is required for the multiple genetic hit mechanisms for cancer development. As an initiator, this UVB creates a mutant cell, which can drive clonal expansion of the single mutant cell later on. The other function for sunlight, including both UVB and UVA, is to induce apoptosis by melanin photosensitization reactions. This will create the opportunities to investigate new non-mutational pathways and to accelerate the clonal expansion step of multiple genetic hit carcinogenesis [48].
Chapter 5

Application and Calculations

5.1 Justification and Application Approach

Since the mid-1980s, a stream of researchers -- such as Marks, Kricker, Kreamer, Ziegler, Laffell, English, Jonason, Nakazawa, Ouhtit, Ren, Yamasaki, Pontén and Brash, among others have reported that the distinctive mutations initiated by UV light allowed early events inferred from mutations observed in tumors [8, 10-12, 15-17, 20-21, 36, 43, 54-59, and 61-68]. In other words, they furnished new insight into the link between sun exposure and skin cancer, and analyzed events occurring between the initial sun exposure received before the age of 18 by normal individuals and subsequent skin cancer occurrence years later [8, 12, 20, and 73].

For instance, research by Jonason et al. has shown that since keratinocytes are continuously lost through squamous differentiation, it is likely that the cell which is targeted by sunlight decades before the tumor’s appearance is a stem cell [8]. Accordingly, the study suggests that the keratinocytes contain the mutations measured in normal skin reside in clonal patches originated from mutated stem cells [8]. This coincides with Gostjeva-Thilly’s study of bell-shaped nuclei and the fetal-juvenile mutator stem cell hypothesis, which states that both net stem cell growth and discernible point mutation

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appear to have ceased at the juvenile-adult transition (around 15 to 18 years of age), and
are not affected by the age, gender, or smoking or non-smoking status [1-2, 5].

Accordingly, three approaches will be taken for this chapter:

(1) In order to apply Gostjeva-Thilly's age independent hypothesis to this current study on
skin cancer, this chapter will first revisit and reanalyze research by Brash, Poten, Zielgler
et al. on the influence of age on frequency and size of epidermal p53 clones via Jonason et
al.'s study on “Frequent clones of p53-mutated keratinocytes in normal human skin” [8].

(2) In order to examine Jonason et al.'s presented data, I will re-calculate their data related
to the patch frequency with respect to age, as well as with respect to the three categories of
sun-exposed data: sun-shielded, intermittently sun-exposed and chronically sun-exposed in
a graphed format to compare with Jonason et al.’s findings [8].

(3) In the “Discussion” section, this study will infer to Gostjeva-Thilly’s fetal-juvenile
mutator stem cell hypothesis. By utilizing Jonason et al.’s data, my calculation presented
in graph format will confirm that both Jonason et al.’s explanation on the age
independence of clone frequency, and Gostjeva-Thilly’s hypothesis indicating that
mutations rarely occur in adult maintenance stem cells that occur at significant constant
rates in pre-adult stem cells involved in fetal and juvenile growth [1-2, 5].

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5.2 Example On "Frequent Clones of p53-mutated Keratinocytes in Normal Human Skin" by Jonason et al [8]:
This portion of 5.2 is based on Jonason et al's findings.

5.2.1 Materials and Methods:

1. Jonason et al. devised a whole-mount preparation method for human epidermis collected from volunteers, none of whom had skin cancer. They used this method to assist immunohistochemical analysis for stabilized p53 protein. Usually a p53 mutation could lead to nuclear immunopositivity.

2. DNA amplification and sequencing:
   DAB-stained patches were microdissected with 30 gauge steel needles, while the total genomic DNA was amplified to a copy number sufficient for multiple p53-specific PCRs. Furthermore, reamplification for exons of the p53 gene was performed with primers, amplification condition, and controls for contamination and polymorphisms, as were direct DNA sequencing of uncloned PCR products and sequence confirmation.

3. Statistical analysis:
   In this area, statistical analyses were performed with patients as the units of analysis.
   a. Using Wilcoxon rank sum test for comparisons of patch frequency and patch size between 2 levels of sum-exposure.
   b. Using the Jonckheere-Terpstra test to perform the test of dose-response.
   c. All "P" values were two-sided.

5.3. p53-Mutant Clones, Sun Exposure, Clone Number, Size, and Frequency

5.3.1 p53-Mutant Clones

The results of the experiment by Jonason et al. are as follows:

Many p53-immunopositive patches are visible by light microscopy. These patches are seen in both the dermal-epidermal junction and the hair follicle, as shown in Figure 9:
Figure 9:

*p53-immunopositive clones in whole-mount preparations of human epidermis.* Clones arising from (A): the dermal-epidermal junction, and (B): a hair follicle. In both cases, view is from the basal surface to show follicles. (x100.)

From *PNAS 93: 14026 (1996)* by Jonason et al. [8]

The dose-response of patch frequency is statistically significant, \( P = 0.0001 \). The difference between each pair of sun-exposure categories is \( P = 0.001 \) to 0.01. Three categories of clone frequency: Sun-shielded, Intermittently Sun-Exposed and Chronically Sun-Exposed – ranged from an average of 3 patches per cm\(^2\) in sun-shielded skin to 33 patches per cm\(^2\) in chronologically sun-exposed area, as shown in **Figure 10:**
Furthermore, it presents that the patch frequency is independent of age. Two distinct morphological patterns are presented: diffused and compact.

In order to determine whether the patches are in fact composed of p53-mutated cells, Jonason et al. microdissected 16 stained compact patches from sun-exposed skin, randomly amplified the entire genome, and then amplified specific exons of the p53 gene. Following direct DNA sequencing of uncloned PCR products, they report that 50% of the patches contain a mutation in the p53 structural gene as shown in Figure 11 below:
Figure 11:

Patches of p53-mutated keratinocytes in human epidermis by Jonason et al:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Location</th>
<th>Patch frequency</th>
<th>Normal sequences</th>
<th>Base change</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun-avoided</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC5A</td>
<td>24</td>
<td>Inner arm</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC17A</td>
<td>36</td>
<td>Abdomen</td>
<td>4</td>
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<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>34</td>
<td>Breast</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>20</td>
<td>Lower abdomen</td>
<td>3</td>
<td></td>
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</tr>
<tr>
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<td>Breast</td>
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</tr>
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<td></td>
</tr>
<tr>
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<td>Lower abdomen</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Breast</td>
<td>8</td>
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<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>YC10H1</td>
<td>24</td>
<td>Upper back</td>
<td>11</td>
<td>tCCg</td>
<td>CC → TT/wt</td>
<td>288 Arg → Gin</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>tCCg</td>
<td>CC → TT/wt</td>
<td>288 Arg → Gin</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td>CC → TT/wt</td>
<td>288 Arg → Gin</td>
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<tr>
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<td>Post-auricular</td>
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<td>C → T/wt</td>
<td>206 Gly → Gin</td>
</tr>
<tr>
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<td>Right thigh</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
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<td>Lip</td>
<td>46</td>
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<tr>
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<td>Pre-auricular</td>
<td>23</td>
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<td>362 Stop</td>
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<td></td>
<td></td>
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<td>YC7E</td>
<td>48</td>
<td>Pre-auricular</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC15A</td>
<td>47</td>
<td>Upper eyelid (right)</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC15B</td>
<td>31</td>
<td>Upper eyelid (left)</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC15A</td>
<td>48</td>
<td>Lower eyelid</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC15B1</td>
<td>48</td>
<td>Lower eyelid (right)</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC15B2</td>
<td>48</td>
<td>Lower eyelid (left)</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC1A</td>
<td>98</td>
<td>Lower eyelid (right)</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC1A</td>
<td>98</td>
<td>Lower eyelid (left)</td>
<td>37</td>
<td>tCCg</td>
<td>CC → TT/wt</td>
<td>288 Arg → Gin</td>
</tr>
<tr>
<td>YC1H2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC1H3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC1B4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC22A</td>
<td>56</td>
<td>Upper eyelid (right)</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC22B</td>
<td>56</td>
<td>Upper eyelid (left)</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC18A</td>
<td>54</td>
<td>Pre-auricular</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YC1A</td>
<td>56</td>
<td>Upper eyelid</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each VC number denotes a different individual; letters denote different tissues, and decimal numbers denote sequenced patches. Both compact and diffuse patches were counted. Underline sequences are for the strand containing the mutating pyrimidine, "wt" denotes the presence of a wild-type allele alone or with a mutant allele.

They also reported that nonstaining skin flanking the patches is wild-type, indicating that each p53 mutation is limited to a patch. The intensities of the mutant and wild-type DNA sequencing bands are equal, indicating the entire patch is occupied by heterozygous mutations, and each patch is a clone.

In comparison, these results are similar to the report from Ren et al. in "Two distinct p53 immunohistochemical patterns in human squamous-cell skin cancer, precursors and normal epidermis" [52]:

1. The dispersed pattern shows no correlation to the age of the individual;
2. The presence of p53 immunoreactivity as a compact pattern supports the theory that mutations of the p53 gene are early events in the sequence from dysplasia to invasive squamous-cell cancer of the skin.

In addition to Ren et al.'s, various publications cited in the beginning of this chapter echo a similar assessment of the influence of age on frequency and size of epidermal p53 clones.

5.3.2 Number and Size of Cells in the Clones

Jonason et al. stated that in order to estimate the number of cells in these clones, they viewed them from the basal surface and counted $\approx 6,400$ mutated cells per mm$^2$. These mutant colonies have a size from 60 to 3000 cells, exceeding frequencies of 40 cells per cm$^2$ involving as much as 4% of sun exposed, normal epidermis.
The size of the clone is larger in chronically sun-exposed skin than in intermittently-exposed skin, suggesting that sunlight not only initiates but also drives clonal expansion. See following Figure 12:

**Figure 12: Increase of clone size with sun exposure**

*Increase of clone size with sun exposure. Dot histogram shows the area in mm$^2$ of individual p53-mutated patches from sun-shielded, intermittently exposed, and chronically sun-exposed skin. Each point represents an individual clone. From *PNAS, USA* 93: 14028 (1996) [8]*

Furthermore, these cells are arranged spatially as conical clones arising from stem-cell compartments and supporting the idea that stem cells are the cell of origin [8]. These p53-mutant stem cells, while Marks and Kricker note, offer a clear explanation of the ability of childhood sun exposure to affect adult cancer frequency [12, 20].
**EPU size and stem cell compartment.** In the absence of UV, their growth is halted and the clones regress. Interestingly, the initial size of the p53-mutated clone is similar to the size of an EPU, or epidermal proliferating unit (i.e. one stem cell compartment), indicating that the stem cell compartment of the epidermis acts as a physiological barrier to clonal expansion of p53-mutated keratinocytes as reported by Zhang *et al.* [74]. Sustained UV exposure deletes neighboring stem cell compartments by apoptosis, thereby enabling p53-mutated keratinocytes to colonize an adjacent compartment without incurring an additional mutation [74].

Average patch size for each level of sun exposure was taken from the data of Jonason *et al.* According to the Gostjeva and Thilly's fetal-juvenile theory, the sun shielded patches would best represent the preneoplastic lesions that occurred before maturity. The intermediate and chronic patches represent the preneoplastic lesions after having been exposed to a mutagen – the sun. From **Figure 13**, it can be seen that the average size of the sun shielded patch is much smaller than the size of the intermediate or chronic patches, which are about equivalent in size. This finding is consistent with the fact that the sun shielded patches were excised from locations in which the preneoplastic lesion would not have been exposed to growth inducing rays from the sun, whereas the intermediate and chronic patches may have started out with similar sizes as the sun shielded patches, but after prolonged exposure to the sun, consistent with the fact that sunlight can be categorized as a carcinogenic promoter.
To test this hypothesis further, the log₂ of sun shielded cluster size in mm² was graphed versus the log₂ of cluster number based upon the same data from Jonason et al. Figure 14 shows a linear fit of these values ($y = 0.0188x - 0.1149$) with a slope close to zero, indicating the absence of change of cluster number based on cluster size, consistent with the fact that these sun shielded patches were rarely exposed to the sun’s carcinogenic rays.
Similarly, Figures 15 and 16 show a similar graph of the log₂ of cluster size versus the log₂ of cluster number. However, the cluster numbers for the intermittently and chronically exposed patches were divided by the average cluster size for the sun shielded patches. This calculation corrects for the growth of the patches due to sun exposure so that cluster number and size can be compared. The lines of best fit were found for the intermittently shielded (y = 0.9874x – 8.0092) and chronic (0.999x – 8.0566) skin patches. The slopes for these lines are almost linear, suggesting that cluster size increased with amount of sun exposure.

Figure 15:
5.4. **Patch Frequency is Independent of Age**

The age independence of clone frequency presented by Jonason *et al.* is very much in accordance with Kricker’s report on substantial precancerous effects during childhood [20], in consistence with Marks’ human skin precancers report [21], and with Berg’s findings of early p53 alterations in mouse skin carcinogenesis by UVB radiation by immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells [59].

In measuring p53 patch frequency, consideration must be given to the total area of p53-immunopositive clones in a skin sample which constitutes a measure of p53 mutation...
frequency. In other words, the patch frequency is calculated by averaging the clone areas and then multiplying by the number of clones per cm² in the same skin sample.

As reported that the patch size often varied ~ 20-fold in the same person. The area of each measured patch is plotted in the histogram, as seen in the previous Figure 12. Clearly, each sun-exposure category contains a different distribution of patch sizes. Since sunlight plays the pivotal role as the mutagen, the average patch size is larger in chronically sun-exposed skin than in sun-shielded skin ($P = 0.02$). Specifically, Jonason et al.’s calculated that the frequency of patches exceeding 0.05 mm² is 5 times greater in chronically exposed skin (25%) than in sun-shielded skin (5%) ($P = 0.04$ by Fisher’s test), with intermittently sun-exposed skin falling in the middle. The combination of both size and frequency has proved that sunlight also plays a promoter role in the promotion of the clonal expansion of p53-mutant cells [8].

Statistically, the p53 mutation frequency ranged from $10^{-3}$ to $4 \times 10^{-2}$ in sun-exposed skin and $10^{-4}$ to $10^{-3}$ in sun-shielded skin. It is estimated that around 4% of sun-exposed normal epidermis contains p53-mutated keratinocytes [8]. In measuring each category, the frequency of patches ranged from an average of 3 patches per cm² in sun-shielded skin to 33 patches per cm² in chronologically sun-exposed area.

The following Figure 18 is calculated to verify and prove that Jonason et al.’s patch frequency is independent of age report. Data are based on Figures 9, 10, 11, 12. The solid line indicates line regression computed for three aforementioned sun-exposure categories: Sun-shielded, Intermittent, and chronically sun-exposed skin. The blue-colored diamond shape is for sun-shielded, red square for intermittent, and yellow triangle

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for chronically-exposed. The results are consistent with Jonason et al. data presentation for mutant frequency and age independence theory:

**Figure 18:**

![Age Independence and Patch Frequency](image)

It can be seen that the lines of best fit for the sun-shielded \(y = 0.1047 - 0.5624, R^2 = 0.1244\), intermittent \(y = 0.0646x + 10.674, R^2 = 0.2811\) and chronic \(y = -0.1227x + 39.649, R^2 = 0.0579\) sample groups have slopes close to zero, indicating the absence of change in the number of lesions as age of the patient increases. This data corresponds with the fetal-juvenile mutability hypothesis and the conclusions made by Sudo et al. for constant cluster number in the lung irrespective of age.
5.5. Inference to the Fetal-Juvenile Mutability Hypothesis and Discussion

The observations presented by Jonason et al. have provided an analogous to lung cancer and colon cancer studies conducted in the Thilly lab. Jonason et al.’s age independent statistics in reference to normal tumor skin, clonal patches, size and frequency are comparative to the research findings in lung epithelium studies, or colon cancer studies [9, 5, 1]. For example, the age independence of p53 clone frequency and the occurrence of substantial mutagenesis arising during childhood indicating that mutagenicity are proportional to the net cellular growth rate [9]. This growth rate of normal juvenile (0.16 doubling/per year) is very similar to the growth of peneoplastic lesion in smokers (0.17 doubling/year) [9].

In addition, measurements taken from the lung has led Sudo et al. to infer that smoking and aging have no effect on the measured point mutant fractions nor on their numerical distributions in the adult lung epithelium, leading to the conclusion that the point mutations must have occurred before maturity. From this data, it is further suggested that the mutant fractions are constant with age in adults because mutations occur much less frequently in adult maintenance stem cells than in pre-adult stem cells involved in fetal and juvenile growth. Furthermore, Sudo et al. proposed that mutations arose during an exponential increase in stem cell number during human organ growth and development [5].

This has been further observed by Gostjeva-Thilly in their stem cell stages and the origins of colon cancer study [1']. Their calculation confirms that the calculated rate of growth of preneoplastia is equal to the calculated growth rate of the juvenile colon, indicating that the tumor initiation actually blocks the development process by which
growing juvenile stem cells are transformed into or replaced by adult maintenance stem cells [1]. In conjunction, Gostjeva-Thilly group’s joint publication reports the discovery of the multiple forms of clear and reproducible nonsperical nuclei present in the fetal gut (see Figure 19 below), colonic adenomas and adenocarcinomas that appear to be absent in normal adult colonic crypts [2]. Named “metakaryotic”, these amitotic cells with stem-like qualities have been observed in high frequency in fetal, preneoplastic and neoplastic tissues of the colon, but not in the adult colon. With non-spherical, bell-shaped nuclei and peculiar “cup-from-cup” forms of symmetrical amitotic fission, these cells also display a form of amitotic asymmetrical nuclear fusion in which multiple nuclear morphotypes populate a fetal organ by emerging from the bell-shaped nuclei [5].

Based on these findings and comparisons, it can be suggested that skin cancer in adults receives its first mutations in the metakaryotic stem cells of the fetal-juvenile state and is able to maintain the positive cellular growth rate of juvenile, capable of developing to preneoplasias [9]. This finding would validate the epidemiological observations, particularly seen in white Australians, in which children but not adults exposed to nuclear radiation or excessive sunlight have greater numbers of tumors per person than their genetic peers that where not exposed to excessive exogenous agents during the fetal-juvenile period.
Figure 19: Nuclear fission of bell-shaped nuclei in the fetal gut

(a and b) Symmetrical nuclear fission: bell-shaped nuclei emerge from bell-shaped nuclei of similar shape.
(c and d) Asymmetrical nuclear fission: a spherical nucleus and cigar-shaped nuclei emerging from a bell-shaped nucleus. Scale bar, 5 μm.


They further stated that such structures might bear on the hypothesis that tumors are a re-expression of embryonic phenotypes, specifically tissue stem cells forming clonal population with derived differentiated cellular phenotypes [2]. These studies published
between 2005 and 2008 [1-2, 5], help to resolve the assumptions and attributions related to skin cancer’s cell of origin, stem cell of fetal organogenesis, adult maintenance stem cells, stems of juvenile growth and stem cells of fetal organogenesis relating to sunlight initiation, promotion, mutant cells, clonal size, frequency and age independence and expansion – questions raised by skin cancer researchers from Brash, Pontén, Zigler, Jonason to Marks, or Krickers, et al.

In fact, Gostjeva-Thilly’s study of metakaryotes, bell-shaped nuclei and the fetal-juvenile mutator stem cell hypothesis indicates that cells with bell-shaped nuclei are responsible for net growth and differentiation in the embryonic gut, adenomas, and adenocarcinomas, and fulfill the requirements for post-embryonic stem cells in carcinogenesis [1-2]. In the following table and diagram below, Figure 20 and 21 summarize stem cells and their qualities in colon fetal development and carcinogenesis observed in histopathological specimens, and a pediatric growth charts revealing average juvenile growth to be a simple exponential function of age between 18 months and maturity [1]:
Figure 20:

Bell-shaped nuclei with higher frequency in "undifferentiated" areas within adenocarcinomas:

<table>
<thead>
<tr>
<th>Bell-shaped nuclei qualities</th>
<th>Fetal cyt (5–7 cell)</th>
<th>Adult normal colon epithelial crypts</th>
<th>Preneoplastic lesions (adenoma)</th>
<th>Neoplastic lesions (adenocarcinoma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation status</td>
<td>Stem cells of fetal organogenesis</td>
<td>Adult maintenance stem cells</td>
<td>Stem cells of juvenile growth</td>
<td>Stem cells of fetal organogenesis</td>
</tr>
<tr>
<td>Fraction of tissue nuclei</td>
<td>High approx 30% in crypts</td>
<td>Rare (approx 2 x 10^-5)</td>
<td>Low (approx 2 x 10^-5)</td>
<td>Relatively high 1% in undifferentiated tumor sectors</td>
</tr>
<tr>
<td>Net growth rate</td>
<td>Approximately 20 doublings/yr</td>
<td>Zero</td>
<td>Very low</td>
<td>Approximately 20 doublings/yr assuming 2 h for nuclear division</td>
</tr>
<tr>
<td>Symmetric divisions</td>
<td>Frequently observed in fetal gut (Fig. 3A)</td>
<td>None observed</td>
<td>No nuclear divisions in approx 2000 bell-shaped nuclei</td>
<td>Frequently observed in undifferentiated tumor sectors (Fig. 3 G)</td>
</tr>
<tr>
<td>Asymmetric divisions</td>
<td>Frequently observed in fetal gut (Fig. 3E)</td>
<td>None observed</td>
<td>Several observed (Fig. 3 K)</td>
<td>Frequently observed giving rise to multiple nuclear morphotypes (Fig. 3 J)</td>
</tr>
<tr>
<td>Nuclear alignment</td>
<td>Linear &quot;head to toe&quot; array in syncytium</td>
<td>Rare nuclei in crypts align with crypt lumen</td>
<td>Alignments differ with crypts in crypt base (Fig. 3 G, H)</td>
<td>Alignments differ as in adenomas but vinytsa with &quot;head-to-toe&quot; alignment of nuclei are evident</td>
</tr>
</tbody>
</table>


Figure 21: Pediatric growth charts:

For 2 Pediatric growth charts for male (A) and female (B) children of the US circa 1995 plotted on log-linear coordinates. These data reveal average growth and height to be a simple exponential function of age between 18 mo and maturity [4].

Specifically, several key points related to skin cancer can be inferred to the Gostjeva-Thilly hypothesis [1]:

1. Tumor initiation and promotion issue: can be explained by Gostjeva-Thilly’s discussion on growth, differentiation in fetal, neonatal, and juvenile development.

2. Mutation of preadult stem cells: are embodied in these bell-shaped nuclei as observed in fetal tissue and tumor samples.

3. Initiated “juvenile” stem cells: can be explained by the continuation to create local patches of juvenile tissue, later on observed as polyps.

4. Mutator phenotype in normal juvenile tissue, observed in these bell-shaped nuclei express: can be explained as maintained in preneoplastic lesions.

5. The slow growth of the preneoplastic colony: can be explained by at least one of the preneoplastic juvenile stem cells to a fetal stem cell phenotype that rapidly creates a lethal tumor mass.

6. Testing the hypothesis tool for tumor initiation occurring at higher rates in juvenile stem cells than in adult maintenance stems: can utilize MAMA, PCR, CDCE technologies originated from the Thilly Lab.

Accordingly, two key questions raised in this study are responded by the inference to Gostjeva-Thilly’s fetal-juvenile mutator stem cell hypothesis:

1. The keratinocytes containing the mutations measured in normal skin reside in clonal patches originated from mutated stem cells: Gostjeva-Thilly explain that the stem-cell mutation limited to the fetal-juvenile period. In addition, their report also indicates that a stem-like cell form in multiple human fetal organs suggesting that point mutagenesis of the epithelia of both the skin and lung are restricted to the stem cells of the fetal-juvenile period [5, 1-2].

2. Age independence issue: Gostjeva-Thilly explain that point mutant fractions are constant with age because mutations rarely occur in adult maintenance stem cells but frequently occur at constant rates in the transition cells of the turnover unit. Consequently, the age-invariant total nuclear mutant fractions and their numerical distributions as clusters are not accounted by a steady state process of mutations in transition cells of adult lung epithelial turnover units. [5]. In addition, The average rate of p53 gene inactivating point mutation per stem cell gene copy is calculated to be \(-1.6 \times 10^4\) per stem cell doubling. Both net growth...
and discernible point mutation appear to have ceased at the juvenile-adult transition and are not effected by aging, gender [5, 1-2].
Chapter 6

Remarks and Conclusion

Cancer is a disease caused by multiple genetic hits in which mutations play a pivotal role. These multiple genetic hits models study the evolutionary process of tissue cells in an individual’s organ from a normal stage to an initiated pre-neoplastic stage, and finally promote to a neoplastic stage that results in tumorigenesis [4]. Furthermore, the implication of these models can predict that normal individuals shall have stable populations of cancer-prone, but noncancerous, mutant cells awaiting for further genetic hits [8].

In the case of skin cancer development, epidemiological and molecular data clearly indicate that sunlight is a carcinogen and the primary cause of skin cancer in which forms of point mutation associated with ultraviolet radiation, and acts as both a tumor initiator and a tumor promoter by favoring the clonal expansion of p53 mutated cells [10, 17].

Addressing these premises in terms of the distribution of mutant cells in human skin along with the exploration of the fetal-juvenile mutability hypothesis, this study has been approached from three angles. First, in order to support the idea that sunlight’s dual roles provide a selective growth advantage to both endogenous and exogenous mutant, this study utilizes Jonason et al.’s report [8], which indicates that normal human skin contains multiple colonies of keratinocytes that stain for aberrant p53 protein. These mutant colonies are larger in size and higher in frequency in sun-exposed skin than in sun-shielded
skin, suggesting that sunlight both induces p53 mutations and stimulates the expansion of mutant colonies. These mutant colonies have an average size of 60 to 3000 cells, exceeding frequencies of 40 cells per cm$^2$ involving as much as 4% of sun exposed and normal epidermis. In addition, since the patch size often varied 20-fold in the same individual, Jonason et al.'s observation is in accordance with Thilly group's claim regarding large variances of mutant cluster sizes (in the normal lung epithelium) [5, 8].

Second: The age independence of clone frequency presented by Jonason et al. is very much in accordance with Kricker's report on substantial mutagenesis during childhood [20], and consistent with Marks' human skin precancers report [21] and Berg's findings of early p53 alteration in mouse skin carcinogenesis by UVB radiation in clusters of preneoplastic epidermal cells [59]. Other quantitative detections of p53 gene mutation in normal skin studies conducted by Nakazawa, Ouhtit, Yamasaki et al. [54-58], or mitochondrial DNA deletions in human skin by Birch-Machin [75], all indicate that no statistically significant association could be demonstrated between p53 mutation frequency and age, gender, sun sensitivity, or total exposure to the biopsy site. In view of the extremely high mutation frequency in his study, Nakazawa et al. suggest that selective clonal expansion of mutant cells may have occurred in vivo [57]. In short, Jonason et al.'s report on the high frequency of aberrant p53 clones in human skin is independent of age among adult. My data analysis in has also verified his theory.

Third: The distribution of mutant cells in human skin, along with the first and second remarks numerated above, are justified by Gostjeva-Thilly's fetal-juvenile mutability hypothesis.
Study conducted by Jonason *et al.* further indicates that (i) the cell which is targeted by sunlight decades before the tumor’s appearance is a stem cell; and that (ii) the keratinocytes contain the mutations measured in normal skin reside in clonal patches originated from mutated stem cells [8]. These two items can be verified by the Gostjeva-Thilly’s study of bell-shaped nuclei and the fetal-juvenile mutator stem cell hypothesis, which states that both net growth and discernible point mutation appear to have ceased at the juvenile-adult transition and are not affected by the age, gender, or smoking or non-smoking status [5, 1-2],

In conclusion, both the observation of Jonason *et al.* in nuclear mutation in the skin and Gostjeva’s report of a stem-like cell form in multiple human fetal organs suggest that point mutagenesis of the epithelia of both the skin and lung are restricted to the stem cells of the fetal-juvenile period. Finally, the impact of Gostjeva-Thilly’s report on the discovery of amitotic “metakaryotic” cells with stem-like qualities in fetal and preneoplastic tissues of the colon cannot be underestimated in cancer research.
References


