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Senescence-like Phenotypes in Human Nevi

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Summary

Cellular senescence is an irreversible arrest of cell proliferation at the G1 stage of the cell cycle in which cells become refractory to growth stimuli. Senescence is a critical and potent defense mechanism that mammalian cells have to suppress tumors. While there are many ways to induce a senescence response, oncogene-induced senescence (OIS) remains key to inhibiting progression of cells that have acquired oncogenic mutations. In primary cells in culture, OIS induces a set of measurable phenotypic and behavioral changes, in addition to cell cycle exit. Senescence-associated β -Galactosidase (SA- β -Gal) activity is a main hallmark of senescent cells, along with morphological changes that may depend on the oncogene that is activated, or on the primary cell type. Characteristic cellular changes of senescence include increased size, flattening, multi-nucleation, and extensive vacuolation. At the molecular level, tumor suppressor genes such as *p53* and *p16INK4a* may play a role in initiation or maintenance of OIS. Activation of a DNA damage response and a senescence-associated secretory phenotype could delineate the onset of senescence. Despite advances in our understanding of how OIS suppresses some tumor types, the *in vivo* role of OIS in melanocytic nevi and melanoma remains poorly understood and not validated. In an effort to stimulate research in this field, we review in this chapter the known markers of senescence and provide experimental protocols for their identification by immunofluorescent staining in melanocytic nevi and malignant melanoma.

Keywords

nevus; oncogene; senescence; DNA damage; melanoma; immunofluorescence; cancer; protocol; Galactosidase; p16INK4A; p53; BRAF

Introduction

Cellular proliferation is highly regulated and key failsafe mechanisms prevent the unwanted expansion of cell number that can lead to cancer. Tumorigenesis may be constrained through two major mechanisms: apoptosis or cell cycle exit, including transient cell cycle arrest, quiescence and senescence (1). Apoptosis (programmed cell death) is a well-described pathway in humans involving energy-dependent biochemical processes that ultimately lead to the cell's own "suicide". This process results in membrane blebbing, pyknosis, and

eventual removal through the immune activity. Quiescence is defined by reversible cell cycle exit and an extended period of metabolic inactivity (2). Senescence, on the other hand, is an irreversible arrest that restricts cellular proliferation in response to physiological stress signals, mitogen levels, or nutrients which would otherwise trigger cell duplication (3–5).

Senescence was first identified in cells with shortened telomeres and telomeric dysfunction resulting in premature cell cycle arrest (6). Subsequently, it was shown that overexpression of an oncogene, *RAS*, could induce senescence in cultured human cells through a telomerase-independent mechanism (7). Numerous studies have since corroborated these findings, now commonly referred to as oncogene-induced senescence, or OIS. OIS is thought to occur when the aberrant activation of oncogenes (e.g. *RAS*, *MYC*, etc.) leads to depletion of cellular energy and replication reserves. The excess oncogenic load causes stalled replication forks and reactive oxygen species-induced damage, activating the cellular senescence program (3–5).

Melanocytic nevi were initially considered to be an excellent example of senescence *in vivo*. These pigmented lesions are viewed as precancerous, and result from a focal and limited proliferation of melanocytes that is driven by the mutational activation of an oncogene such as BRAF. *BRAF*^{V600E} is the most common oncogenic mutation found in human melanomas (8, 9). After forming a nevus, nevomelanocytes undergo a permanent exit from the cell cycle that prevents them from progressing to melanoma (10, 11). In support of this model, primary cultured melanocytes transduced with a *BRAF*^{V600E} expressing lentivirus experience an initial burst of proliferation followed by morphologic changes and increased levels of SA- β -Gal (10). These changes are one of the most obvious hallmarks of senescent melanocytes in culture and include loss of the elongated fusiform profile characteristic of highly proliferative melanocytes, gain of a flattened egg-shape, and an appearance of multinucleation and vacuolation. On the other hand, nevomelanocytes present in common acquired melanocytic nevi have not been reported as morphologically distinct from adjacent normal skin melanocytes when examined histologically. Rather, it is the presence of nesting, or clustered growth of melanocytes, that distinguishes junctional nevi from solar lentigo. Analysis of the morphology of melanocytes in normal skin and in nevi using confocal microscopy on thick sections (50 μ m), and some of the antibodies and markers described here, will allow characterization of any true senescent features of nevomelanocytes.

Beyond morphology, numerous molecular markers of senescence have been utilized in various cell types that may be also useful for the study of senescence in nevus and melanoma formation. Senescent cells, typically those with a flattened egg-shape and a vacuole-rich cytoplasm, exhibit unusually high lysosomal β -Gal activity (2). Some have speculated that oncogene activation in the presence of intact tumor suppressors may cause cell hypertrophy and subsequent compensatory activation of lysosomal enzymes, including β -Gal (11–12). β -Gal gene silencing experiments demonstrated that its expression functions as a marker or an indicator of senescence rather than as a contributor to the process (13). Additionally, DNA damage (p53, γ -H2AX), proliferation (Ki67 and C-MYC), cell cycle regulation (p16INK4A, p14ARF, p15INK4B, FBX031 and p53) and chromatin structural changes (Senescence-Associated Heterochromatin Foci or SAHF) indicators were used in melanocytes and other cell types as putative senescence markers (2, 14, 15). A tissue

specific hyper-secretory phenotype was also identified in senescent cells (16–18). A list of markers used in the literature is presented in Table 1. To validate the utility of some of them in distinguishing nevi (senescence) from melanoma (senescence bypass or escape), we performed immunofluorescent staining on paraffin sections from 10 nevus and 10 melanoma samples. We selected Ki67 (proliferation) and γ -H2AX (DNA repair foci) as representative biomarkers for senescence (Figure 1), and we observed successful staining using the protocol provided below. Positive staining for these proteins was detected in both nevus and melanoma samples, although it was clear that melanomas had considerably higher proliferation and DNA repair foci. We also observed successful staining for β -Galactosidase using the β -Gal antibody (ab9361; Abcam; data not shown), which may provide an alternative to the SA- β -Gal biochemical assay that requires the less commonly available frozen sample compared to paraffin-embedded tissues generated in most pathology practices. MART-1 is used in clinical diagnosis and identifies melanocytes and melanoma cells. Since known molecular indicators of senescence are not exclusive to this process, the use of multiple markers may be necessary for identification of senescence *in vivo*.

There is a great need for further studies in this area, since conflicting reports have questioned the sensitivity and specificity of these biomarkers for recognizing senescent nevus cells. Several publications could not distinguish between nevi and melanoma using some of these reported markers (15, 19, 20). Only Ki67 appeared to consistently distinguish human nevi from a panel of primary and metastatic melanomas. Likewise, p16INK4A is reliably used in clinical diagnosis to distinguish between nevi and melanoma. It is possible that there are differences between *in vivo* with *in vitro* biomarkers of senescence. Alternatively, nevi and melanoma are not necessarily mutually exclusive, since approximately 25% of melanomas appear in pre-existing nevi (20–22). Heterogeneity of cell properties and mutation content is a well-established idea for tumors that has not been considered in reference to nevus senescence and evasion or bypass of senescence. The concept that nevi are irreversibly senescent has not been well documented, particularly since low mitotic activity has been found in congenital, common acquired, and dysplastic nevi (18, 22). It is anticipated that quantitative differences may be observed between nevi and melanoma, necessitating studies that consider staining patterns in greater detail. If senescence-like phenotypes can be reliably identified in nevi, it will lead to improvements in histological detection of melanoma and to novel avenues for anti-melanoma therapy.

Materials

- **Primary Antibodies:** Ki67 (VP-K452; Vector), γ -H2AX (20E3; Cell Signaling), p16^{INK4a} (N20 and C20; Santa Cruz), MART-1 (18-7263; Invitrogen), β -Gal (ab9361; Abcam)
- **Secondary Antibodies:** Secondary anti-IgG antibodies conjugated with Alexa Fluor 594 and Alexa Fluor 488 (Invitrogen)
- 100%, 95% and 70% Ethanol
- Xylene (247642-4L-CB; Sigma-Aldrich)
- Sodium Citrate Buffer 10X, pH 6.0 (C9999;Sigma)

- Pressure Cooker (CPC-600; Cuisinart)
- **PBST:** 0.1% surfactant Tween-20 (BP337-500; Fisher), and 1XPBS (D8537; Sigma-Aldrich)
- **Blocking Solution:** 10% γ -globulin free BSA/PBST/2 % normal immune serum: Add 100 μ L of normal goat serum (S-1000; Vector) to 4.9 mL BSA/PBST
- Hydrophobic pen (Immedge; Vector)

Methods

1. Dewaxing sections

1. Place slides in a dipping rack and transfer successively to 3 plastic jars containing xylene while incubating in each for 5 minutes (See Notes 1, 2).

2. Rehydrate slides

1. Bring to absolute alcohol by placing the slide rack in 100% EtOH baths twice for 5 minutes each.
2. Start the rehydration by transferring successively the slide rack to two jars containing 95% EtOH 5 minutes each time then into two successive 70% EtOH consecutive baths twice for 3 minutes each.
3. Place slide rack in water then under slowly running water for 2 minutes.

3. Pressure Cooker Antigen Retrieval

1. Pour 10mM Sodium Citrate, pH 6.0 into heat-resistant coplin jar (See Notes 3, 4).
2. Load slides into the citrate filled slide containers, close lid and put in the pressure cooker.
3. Prepare the pressure cooker by adding water and place the plastic coplin jar with slide containers into the cooker (See Note 5).
4. Start pressure cooker on HIGH setting: P=10psi, T=201-220°C for 15 min (See Note 6).
5. Remove slide containers and cool down for 20-30 min at room temperature.
6. While cooling, prepare blocking solution (described below).

¹Allow racks to drip when transferring between the plastic jars to reduce carry over to keep concentrations in the contents of the jars unchanged and minimize

²Keep xylene and EtOH tubs clean by replacing every four to five runs.

³Allow an even distribution of slides in the citrate container for best epitope retrieval.

⁴Tap each slide once or twice before placing in the Sodium Citrate solution. Excess water carry over from the slides can alter the pH of the buffer.

⁵To save time, fill the pressure cooker with a few cups of water and set to "Keep Warm" while rehydrating the slides.

⁶Ensure the pressure cooker is in fact holding pressure. If so, there should be no steam leaking from the cooker.

7. Transfer slides to a rack in a plastic jar containing water and wash under running water for 3 minutes.
8. Transfer slide rack to two PBS wash tubs, for 5 minutes each.

4. Blocking Background

1. Prepare a humid chamber to prevent evaporation of blocking solution and later on the primary antibody. A large slide box with a layer of moist tissue paper or a pool of water in the bottom could be used to create the moist chamber.
2. Take slide out of 1XPBS, dry back of slide with a Kimwipe, gently dab front of slide around tissues, and aspirate PBS from around sample carefully (Do NOT touch the tissue!)
3. Delineate each tissue sample with a hydrophobic pen to help retain the reagents on the section.
4. Add 1XPBS onto slide immediately so they don't dry out.
5. Aspirate carefully the PBS and immediately replace with the blocking buffer to cover each section completely. This step will block sites of non-specific affinity for immunoglobulins (See Note 7).
6. Close the humid chamber and incubate for one hour at room temperature.

5. Primary Specific Antibody

1. Quick vortex the antibody stock tube. Dilute the specific antibody in the blocking solution. *Dilution ratios change depending on which antibody is used and are normally provided by the manufacturer.* If double staining is desired, add both primaries at the same time.
2. Label slides at this point with (–) no primary antibody (control) and (+) primary antibody to be added (See Note 8).
3. Aspirate the blocking solution from the (+) –labeled sections and leave the (–)–labeled sections intact (See Note 9).
4. Add just enough diluted antibody to cover the (+)–labeled tissue sample.
5. Incubate in closed humid chamber overnight at 4°C

6. Washes

1. Tap off the primary antibody from each slide onto a Kimwipe and place in a 1XPBS tub.

⁷More solution may be required to fully cover large tissue sections; however, overloading sections is unnecessary and can lead to seepage. Avoid creating bubbles if possible.

⁸By convention the negative control is closest to the label.

⁹Don't allow sections to dry. This is best achieved by working with one slide at a time rather than aspirating, or removing all the slides from PBS, at once.

2. Wash in 1XPBS three times, 5 min each to remove the unbound antibody. In the meantime, prepare the secondary antibody as described below.
3. Aspirate the liquid from both (+) and (–)-labeled sections, and immediately add the diluted secondary antibody (See Note 9).

7. Secondary Antibody

1. Quick vortex the Alexa Fluor-conjugated antibody stock tubes (See Note 10).
2. Dilute the secondary antibody to 1:1000 in blocking solution. If double staining is desired, make sure that each primary antibody is generated in a different species of animal so that the secondary antibodies cannot cross hybridize and that there is no spectral overlap. We chose Alexa Fluor 488 (green) and Alexa Fluor 594 (red) as fluorescent conjugates for our secondary antibodies.
3. Incubate for one hour at room temperature in a humid chamber in the dark.

8. Washes/Coverslip

1. Tap off the primary antibody, stick slides in racks and place these in a plastic jar containing 1XPBS wash.
2. Wash slides 3 × 5 min in 1XPBS to remove the unbound antibody.
3. Remove liquid from all sections and add one drop of permanent mounting medium containing the DNA dye DAPI (intercalates into the DNA) for nuclear visualization.
4. Coverslip and allow an even spread of the mounting medium over the section by applying pressure gently to the slide cover with forceps. Make sure that there are no bubbles formed. If a hardening mounting medium is used, it is best to allow the sample to cure overnight, so next day microscopic examination is performed.
5. Place in slide tray and keep in a slide folder at 4°C until ready to view but keep in mind that the fluorochromes' fluorescence intensity diminishes over time.

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¹⁰After vortexing, collect the secondary antibody from the surface. Denser aggregates of antibody settle at the bottom of the tube and can contribute to background artifact in the staining.

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Figure 1. Hematoxylin and Eosine (H&E) and immunofluorescent staining of biomarkers in human nevus and melanoma sections, 20X. **(A)** H&E staining. In these examples, the nevus shows extensive nesting while the melanoma has a high density of melanocytes at the dermal-epidermal junction. **(B)** MART-1 and γ -H2AX immunostaining labelling melanocytes (cytoplasmic) and DNA damage (nuclear), respectively. **(C)** MART-1 and Ki-67 double

immunostains label melanocytes and detect proliferation, respectively. Double positivity marks proliferative melanocytes.

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Table 1

Summary of literature findings for cellular senescence associated markers.

Marker	Association	Cell/Tissue Studied	Reference
SA-β-Gal	Senescence induction	HUVEC *	(24)
p16 (INK4A)	Cell Cycle Regulation	Melanocytes	(25, 26)
p15(INK4B)	Cell Cycle Regulation	Prostate Tissue`	(20)
ARF (p14)	Cell Cycle Regulation	Melanocytes	(25)
Ki67	Proliferation	Melanocytes	(15)
C-MYC	Proliferation	Cancer Cells	(27)
SAHF **	Chromatin Remodeling	Melanoma Cells	(28, 29)
γ-H2AX	DNA Damage	Melanocytes	(15, 19)
p53	DNA Damage	Melanocytes	(19)
FBXO31	Senescence induction	Melanoma Cells	(26)
IGFBP7	Senescence induction	Nevi, Melanoma Cells	(26)
PML-IV Bodies	Senescence induction	Melanoma Cells	(28)
miR-203	Senescence induction	Melanoma Cells	(30)
Dec1	Senescence induction	Cancer Cells	(31)
DeR2	Senescence induction	Cancer Cells	(32)
RAS^{G12V}	Senescence induction	Retroviral Cells	(33)

* Human umbilical vein endothelial cells (HUVEC)

** Senescence Associated Heterochromatic Foci (SAHF): H3K9Me, HP1, H2A