Original Basic Research

Biogenic amines and tumor-vascular interaction in melanomas

Iyengar Bhanu

Pigment Cell Centre, New Delhi, India

Corresponding author: Dr Bhanu Iyengar,MBBS, MD, PhD, FAMS,Pigment Cell Center,Iyengar Farm,Brijwasan Road,PO Kapashera,New Delhi 110037,India; Tel: 91-11-25063433. E-mail: bhanu_i@yahoo.com

Received: 2011-07-04 **Accepted:** 2011-09-10 **Published:** 2011-10-20
DOI: 10.5430/jst.v1n2p44 **URL:** http://dx.doi.org/10.5430/jst.v1n2p44

Abstract

Background

In nodular melanomas in vertical growth phase, tumor-vascular complexes (TVC), with perivascular mantle zone (PMZ) of 5 to 6 layers (L1-L5) of tumor cells are formed around angiogenic vessels at the tumor-stroma interphase, showing organized neural differentiaton. The present study explores the sequence of biogenic amines positivity in relation to angiogenesis, utilizing the TVC as a 3D model.

Methods

The sequence of positivity for Nestin (Nes), GFAP, indoleamines: serotonin (SER)/melatonin (MLT), catecholamines: dopamine (DA)/noradrenalin (NA), pigment (Pig); dopa oxidase (DO); and related mitosis in layers of the PMZ (L1-L5) was assessed in 897 developing TVCs using 5 μm serial paraffin and frozen sections. Statistical Analysis: ANOVA: Kruskal-Wallis One Way Analysis of Variance; All Pairwise Multiple Comparison Procedures (Tukey Test).

Results

Nes, GFAP appear in L1/L2, next indoleamines (serotonin/melatonin) in L2/L3 associated with mitosis. DA is positive in L3/L4 coinciding with DO which peaks in L4 with NA, Pig appears in L4/L5. Mitosis in L2/L3 is associated with indoleamine positive cells which remain in L2/L3, with catecholamine positive cells moving into L4/L5 thus establishing a polarity.

Conclusion

Thus, the angiogenic vessel confers polarity and an embryonal microenvironment in the PMZ, the aggressive melanoma cells functioning as neuronal stem cells to resemble early neurogenesis of bio-aminergic cells.

Key words:

Biogenic amines; Indoleamines; Catecholamines; Dopaoxidase; Angiogenesis; Nestin; GFAP.

INTRODUCTION

Mammalian melanocytes originate as multipotent neural crest (NC) cells from the neural fold, which lies at the confluence of the neuroepithelium and the general epidermis, that detach from the neural tube to arrive at the dorsolateral surface by day 8.[1-3] These multipotent NC cells give rise to peripheral neurons, glial cells, neuroendocrine cell types, epidermal as well as pigment cells during embryogenesis of the neural tube.[1] Melanocytes are NC derived pigment cells which process tyrosine, expressing catecholamines and the related enzymes. Several studies have shown the expression of indoleamines in melanocytes.[4-7]

A recent study of melanomas shows a regulated pattern of differentiation of tumor cells in relation to angiogenesis. Tumor cells which come in contact with newly formed vessels differentiate into GFAP+ve radial glia like cells. As additional layers are added, a compact tumor-vascular-complex (TVC) is formed, with the outer layers showing neural differentiation with NFP and Syn positivity. [8] The present study explores the expression of biogenic amines in relation to angiogenesis in melanomas, in view of the common origin of the serotoninergic and dopaminergic neurons during neurogenesis. [9]

The purpose of this study is to utilize this 3D model showing patterned neural differentiation in tumor vascular complexes (TVC) in melanomas [8] to study the appearance of the biogenic amines, indoleamines and catecholamines, which have been identified in the marginal melanocytes in vitiligo in the tumor cells. [10-11]

MATERIAL AND METHODS

Melanoma: 27 nodular melanomas in the vertical growth phase (VGP) were received from the Cancer Surgery Unit, fixed in 10% formol glutaraldehyde for overnight cold fixation. 10 blocks were taken from each tumor to include equal number of pigmented and amelanotic nodules. 5 µm thick serial paraffin and frozen sections were subjected to routine histochemistry to assess pigment (Pig), and enzyme histochemistry to assess dopa oxidase (DO); related mitosis; immunohistochemistry for positivity of nestin (Nes), glia fibrillary protein (GFAP), the indoleamines: serotonin (SER) & melatonin (MLT), and the catecholamines: dopamine (DA) & noradrenalin (NA), by the avidin-biotin method using Dako Pat kits. [12-15] As negative control all slides included a Serial section stained with no mAb. The same mAb were used simultaneously against known positive sections from human skin as positive controls.

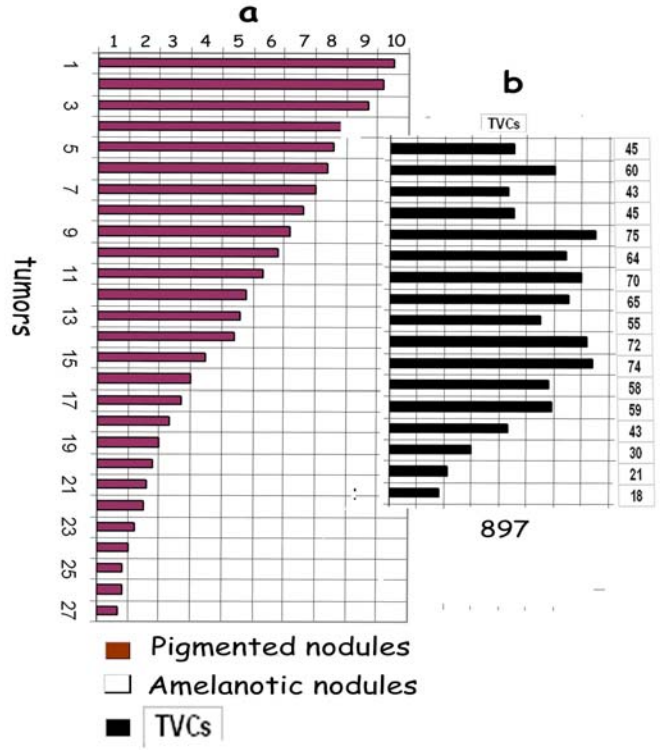

Figure 1 Composite graph showing [a] pigmented and amelanotic nodules sampled in each tumor and [b] the number of TVCs studied at the tumor-stromal interphase, totaling 897.

Tumor-vascular complex

The marginal interphase between the tumor and stroma is included in 65 blocks from the sampled pigmented nodules. These were selected (Figure 1), to study the tumor/vascular interaction during angiogenesis. Interacting angiogenic vessels to a depth of 2HPF from the tumor margin have been included in the study. The formation of the tumor vascular complexes (TVC) around a central angiogenic vessel was assessed in a total of 897 developing complexes and the sequence of appearance of marker positivity recorded. Of the 897 TVCs, 249 show a single layer of tumor cells, 213 show 2 layers, 217 show 3-4 layers while 218 are fully formed with 5 layers.

The distribution pattern of the biogenic amines in relation to the central vessel in the TVC with 5 to 6 layers of tumor cells forming a mantle, the perivascular mantle zone (PMZ) have been taken up for study. For analyses the layers in each TVC are numbered from L1 to L5 with layer 1 being closest to the vessel. Since the sequential positivity is being assessed L1 is present in all 897 TVCs, except for Nes which appears only in the single layer stage, L2 in 648, L3&4 in 435 and L5 in 218 TVCs and the positivity for each marker is collated as percentages for comparison. Positivity for each mAb in each layer is marked out in Camera Lucida (CL) diagrams. The percentage positivity in each layer is mapped onto an aggregate CL figure as well in graphs, in the relation to the angiogenic central vessel.

Statistical Analysis

ANOVA: Kruskal-Wallis One Way Analysis of Variance; All Pairwise Multiple Comparison Procedures (Turkey Test).

RESULTS

As interactions between tumor cells and angiogenic vessels take place within the solid tumor as an integrated, interactive phenomenon, the mantle zones of the TVCs are the best if not the only indicators of the in situ responses, since no definite tissue/organ culture methods are available to study this dynamic process. As the vessels grow tumor cells first accrue and then proliferate along the length. Cross sections of these vessels, on addition of each layer gives a sequential picture.

Tumor cells in contact with newly formed vessels differentiate into GFAP+ve radial glia like cells with the outer layers showing neural differentiation with NFP and Syn positivity. This 3D model has been utilized to explore the appearance of biogenic amines in relation to angiogenesis in view of the common origin of the serotoninergic and dopaminergic neurons during neurogenesis.

Nestin and GFAP

Nes positivity is seen in the L1 at the single layer stage forming 94.5% of layers (235/249 PMZ) beyond which positivity ceases. GFAP positivity is seen in 91.5% in L1 (821/897 PMZ) at the single layer stage forming 93% in L2 (603/648 PMZ), 62% in L3 (270/435 PMZ) and 26.6% in L4 (116/435 PMZ) while L5 is negative (Figure 2a&b,4a).

Of the pooled data there is a significant difference among the treatment groups: On assessment with ANOVA: L1&2: GFAP/nestin positive cells in layers 1 & 2 are significantly higher than NA & DA (F=13.885; P<0.030).

Indoleamines

SER and MLT positivity is higher in the inner layers of the PMZ as compared to the outer layers. SER positivity is 60% (538/897 PMZ) in L1 and peaks in L2 with 73.3% positivity (475/648 PMZ). SER positivity is (46.7%) (203/435 PMZ) in L3, 19.6% (85/435 PMZ) in L4 and 6.7% (15/218 PMZ) in L5, maximum positivity being in L2. MLT positivity is 54.6% (490/897 PMZ) in L1, 64.3% in L2 (417/648 PMZ) and 42.8% (186/435 PMZ) in L3. In the L4 and L5 the positivity decreases being 19.7% (86/435 PMZ) and 9.9% (22/218 PMZ) respectively Thus peak positivity is in the L2 (Figure 2c&d, 4b).

The indoleamines positivity (SER and MLT) was significantly higher than that of the catecholamines (DA and NA) in layers 1&2; (L1: F = 546.83, P <0.001; L2: F = 447.77, P< 0.001). There was no significant difference in the percentage positivity for SER and MLT in L1 (59.67 ± 2.5 vs. 54.6 ± 2), while in L2 the percentage of SER positive cells (73.2 ± 2.1) was higher than those for MLT (64 ± 2.5; P= 0.005. A smaller percentage of L1 (27 ± 2.5) are positive for DA whereas none of the cells in this show NA positivity.

 ISSN 1925-4067 E-ISSN 1925-4075

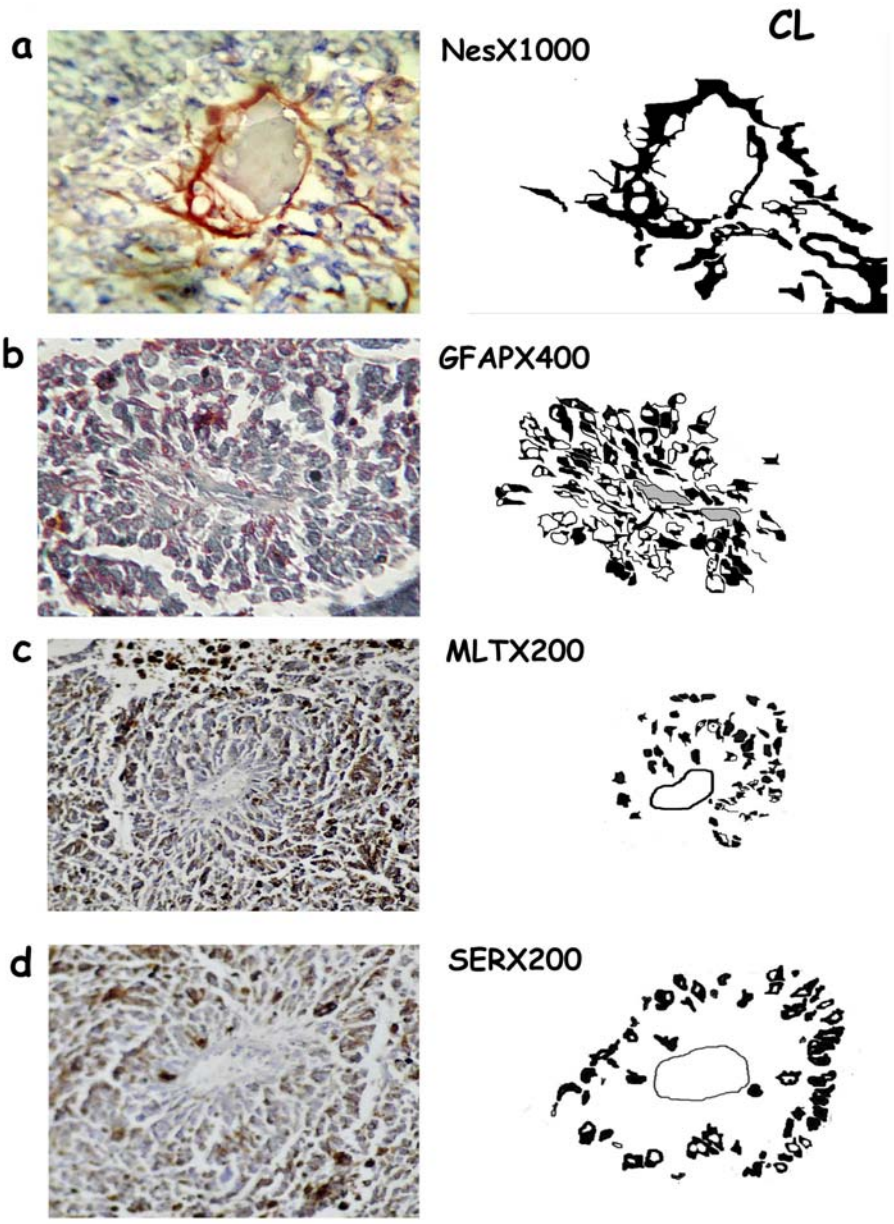

Figure 2 A composite figure alongwith camera lucida [CL] diagrams showing: [a] Nestin positivity in the single layer of cells around the angiogenic vessel. Offshoots of the vessel are seen in the lower left corner[Nes×1000]. [b] An early 2-layered TVC with a central endothelial tube showing GFAP positive cells in L1/L2 assuming a dendritic appearance. Positive cells beyond are rounded[GFAP×400]. [c] A 3-layered TVC with MLT positive cells in L2/L3[MLT×400]. [d] A 2-3 layered TVC showing SER positive cells in L2/L3[SER×400].

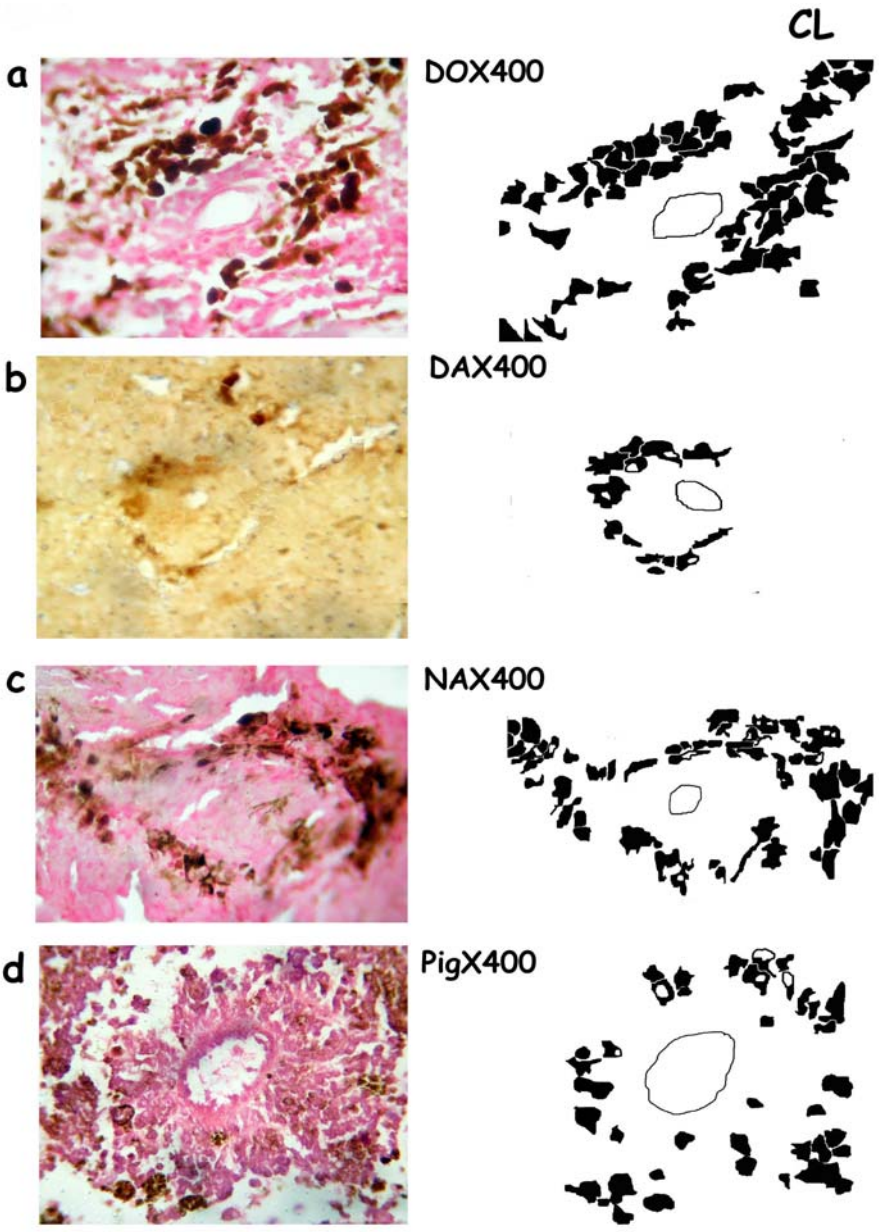

Figure 3 Composite figure with CL diagrams to show catecholamine positivity: [a] Dopaoxidase in L3/L4 in a 4 layered TVC[DO×400]. [b] Dopamine in L3/L4 in a 5 layered TVC[DA×400]. [c] Noradrenalin positive cells are seen in L4/L5[NA×400]. [d] Pigment is seen in the outer layers L4/L5, mostly in L5[Pig×400].

ISSN 1925-4067 E-ISSN 1925-4075

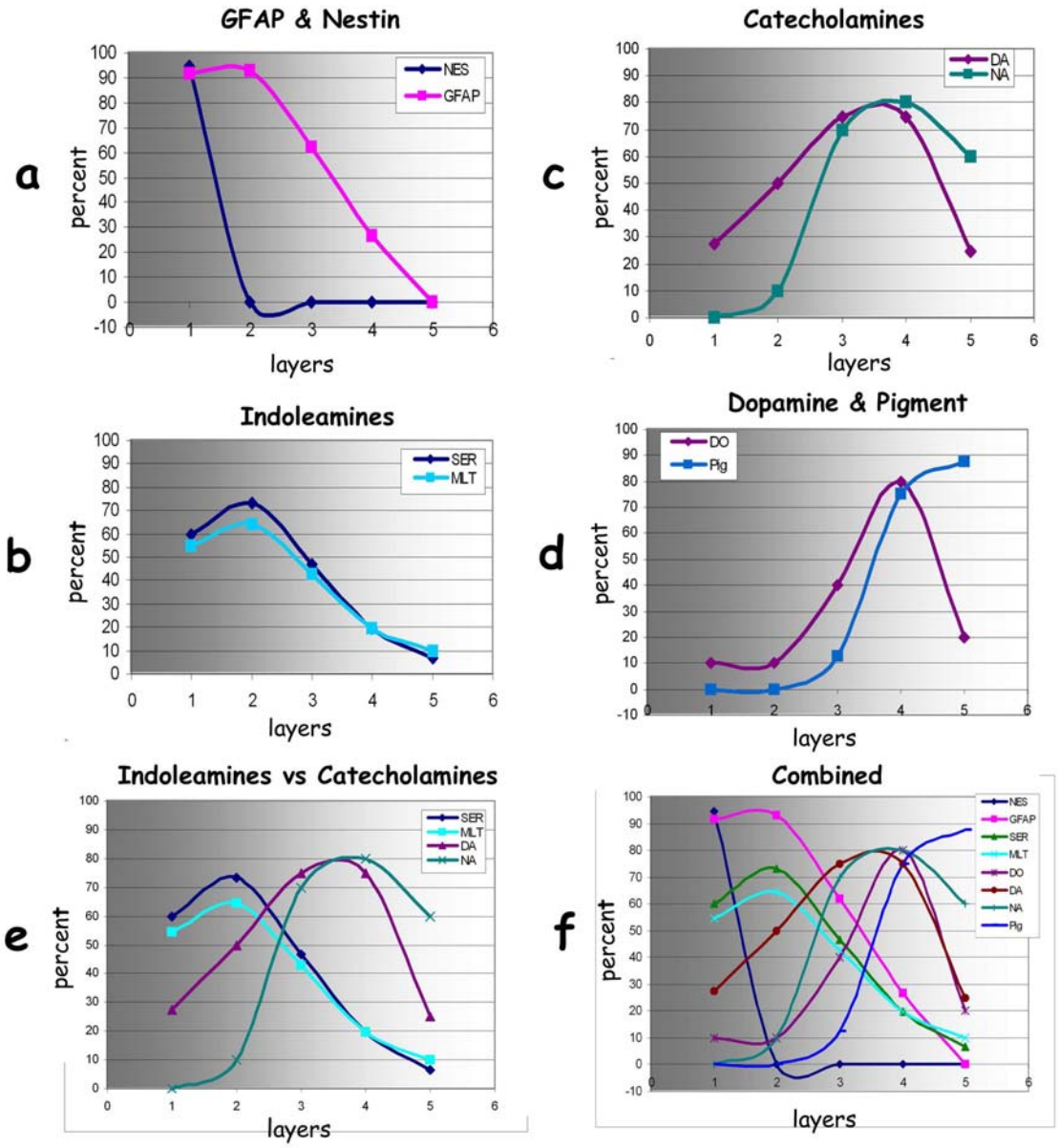

Figure 4 Graphs comparing the positivity of: [a] GFAP/Nestin in L1/L2 [b] Indoleamines in L2/L3 [c] Catecholamines NA/DA in L3/L4 [d] and Dopaoxidase and pigment in the L4/L5. [e] Compares indoleamines with catecholamines, the indoleamines showing peak positivity in L2 while catecholamines are positive in L3/L4. [f] Combination graph to compare the appearance of different substances as the TVC enlarges.

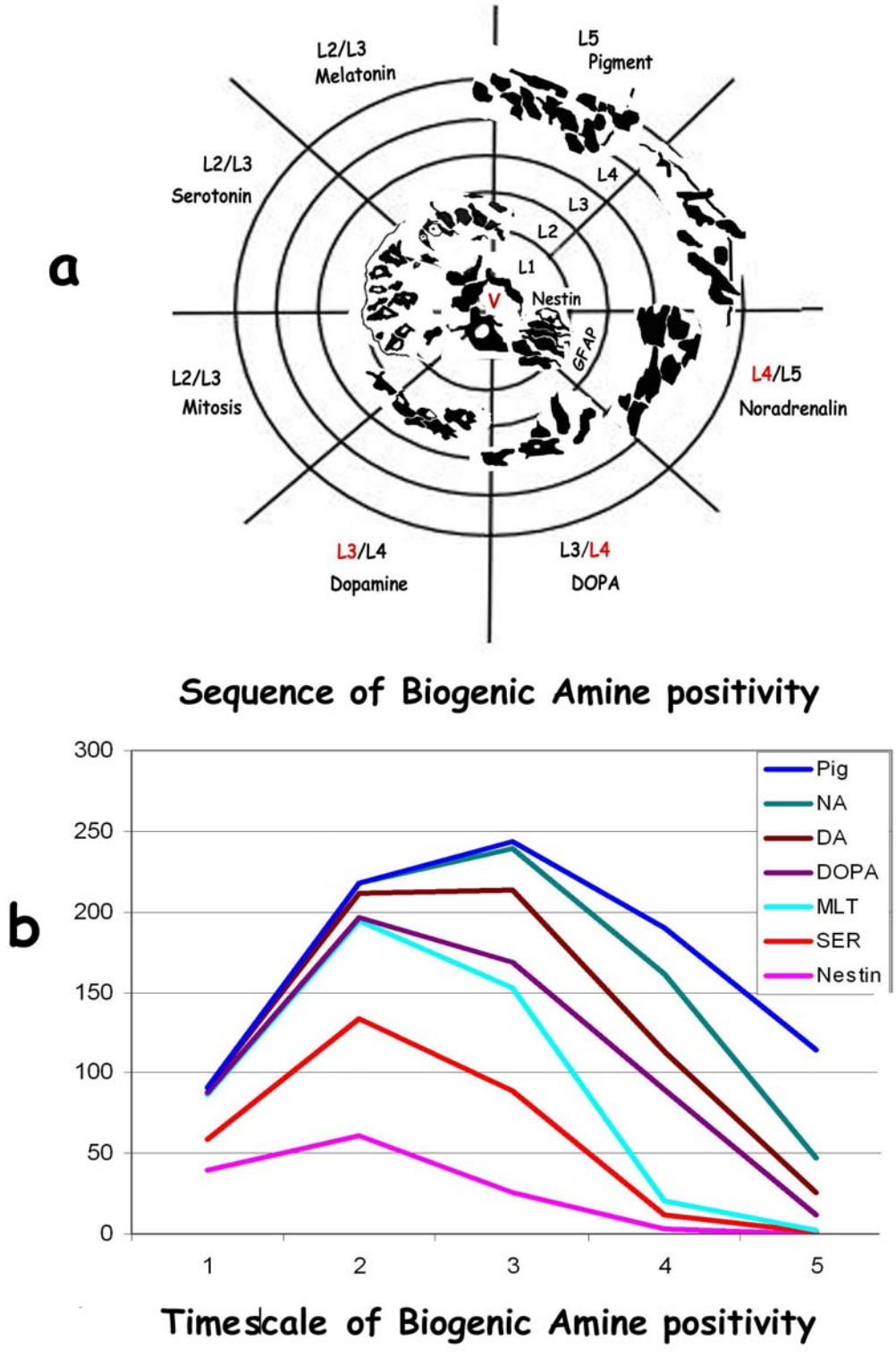

Figure 5 Summary [a] Diagramatic representation of the TVC to show sequential appearance of stem cell markers GFAP/Nestin in L1/L2; indoleamines in L2/L3; catecholamines: DA in L3/L4 and NA in L4/L5; DOPA and pigment in L4/L5. This highlights the polarity established by the interaction of the tumor cells with angiogenic vessels to form the TVC. [b] Time-scale graph showing a graphical depiction of the sequence.

 ISSN 1925-4067 E-ISSN 1925-4075

DOPA oxidase and Catecholamines(Figure 3,4c&d)
DOPA oxidase (Figure 3a,4d) is the crucial triphasic enzyme in catechol metabolism resulting in DA/NA and pigment. Highest DOPA positivity is in the L4 (80%) (348/435 PMZ) . In the inner layers of the TVC the DOPA positivity is 10% (91/897 & 65/648) in L1&2 and 40% (174/435 PMZ) in L3. 20% (44/218 PMZ) layers are positive in the L5.

There were significant differences in dopa oxidase (DO) positivity in different layers of the tumor vascular complex (F 371.60, $P < 0.001$). The percentage positivity for DO was the highest in L4 (80.67 ± 2.1), followed by L3 (40.0 ± 3.0) while positivity was lower in L5 (19.67 ± 3.5) and L2 (10.0 ± 2.0) the lowest positivity being in L1 (9.67 ± 2.5). Pairwise comparisons revealed that the DO positivity in different layers of the tumor vascular complex were significantly different from each other ($P < 0.001$).

DA and NA (Figure 3b&c,4c) positivity is higher in the outer layers of the PMZ as compared to the inner layers. DA positivity is low in the inner layers being 27.5% (247/897 PMZ) in L1 and 50% (324/648 PMZ) in L2. DA positivity (75%) (326/435 PMZ) is in the L3&4, and 25% (55/218 PMZ) in L5, maximum positivity being in L3 & L4. There is no NA positivity in L1, 10% positivity (65/648 PMZ) in L2 and 70% (305/435 PMZ) in L3. In the L4 and L5 the positivity increases being 80% (348/435 PMZ) and 60% (131/218 PMZ) respectively Thus peak positivity is in the L4.

Pairwise comparisons revealed that the percentage of positivity of the catecholamines, DO and pigment was significantly higher than indoleamines in L3, L4 & L5 ($P<0.001$). There were no significant differences between the catecholamines in L3 & L4 (DA, 75.0 ± 3.0; NA, 70.0 ± 2.0) while in L5 DA positivity was significantly lower than NA positivity ($P < 0.001$).

The pattern in L5 was the reverse of that in Layer 1, with SER (5.9 ± 1.6) and MLT (9.6 ± 1.5, $P < 0.001$) positivity being significantly lower than DA (25.0 ± 2.0) and NA (61.0 ± 2.6, $P < 0.001$).

Pigment (Figure 3d, 4d): positivity is higher in the outermost layers of the PMZ as compared to the inner layers. There is no positivity in L1&2, 12.5% (54/435 PMZ) in L3. In the L4 and L5 the positivity increases being 75% (326/435 PMZ) and 87.5% (191/218 PMZ) respectively Thus peak positivity is in the L5.

In comparison, L1 and L2 of the tumor vascular complex were not pigmented. Pigment positivity increased significantly in subsequent layers ($F = 4088.40$, $P< 0.001$). There was a significant increase in the amount of pigment from L3 (11.67 ± 1.4, $P < 0.001$), to L4 (74.67 ±1.5) thence to peak in L5 (87.83 ± 1.5) ($P < 0.001$).

The interesting feature is the separation of indoleamine and the catecholamine positive cells into different layers as seen during neurogenesis.

DISCUSSION

In a recent study, utilizing the TVC as a 3D model, it is seen that tumor cells in VGP melanomas interact with angiogenic vessels to differentiate first into radial glia like multipotent stem cells, followed by Syn and NFP positive cells in a coordinated manner. [8] The present work was taken up to identify neuronal cell types indicated by Syn and NFP positivity. Melanocytes process tyrosine to produce melanin pigment like the pigmented neurons in the brain. Further, serotonin and melatonin are expressed in the skin melanocytes. [7] In view of this, the present work was taken up to study the pattern of expression of biogenic amines in the TVC. The results indicate that the TVC model delineates the differentiation of indoleamine and catecholamine producing cells from GFAP/nestin positive stem cells in relation to angiogenic vessels.

Nestin is the predominant marker used to identify stem and progenitor cells in the mammalian CNS. It is a protein belonging to class VI of intermediate filaments produced in stem/progenitor cells during development, and is expressed mainly in neuroepithelial stem cells but not in mature elements. During neuro- and gliogenesis, nestin is replaced by cell type-specific intermediate filaments, e.g. neurofilaments and glial fibrillary acidic protein (GFAP). Presence of nestin in cells indicates multi-potentiality and regenerative potential. In neural cell cultures 10-30% of the cells population stained for GFAP, and co-immunostained for nestin during the first week. Nestin is expressed in endothelial cells of CNS tumor tissues and of adult tissues during angiogenesis as a marker protein for neovascularization. [16-20]

GFAP, a specific marker for astrocytes of the CNS is a 50 kDa intracytoplasmic protein, constitutes the major cytoskeletal protein in astrocytes. [21] GFAP positivity identifies the radial glial multipotent astrocytic stem cells during embryogenesis as described in several studies. [21,22]

Neurogenesis is a gradual process that transforms undefined neuroepithelial cells into fully differentiated neurons in two phases: an early, premitotic phase when precursor cells proliferate and adopt a more restricted cell fate; and a postmitotic phase, when neurons are fully committed to a specific cell type. [23] During embryogenesis, the serotoninergic and dopaminergic neurones arise from common progenitors. Biogenic amines have common progenitor stem cells with enzymes cat-1 & cat- 4 and bas-1. [24-27] As development progresses, the serotoninergic cells remain at the brain stem and the post-mitotic dopaminergic cells proceed to the midbrain SNC.

Proliferating biogenic amine progenitor cells are specified by the combined actions of two signaling proteins, sonic hedgehog (Shh) and fibroblast growth factor 8 (Fgf8). The combined signaling by these two proteins leads to the induction of the progenitor cells[24]. The mesencephalic DA neurons are generated in the immediate vicinity of two organizing centers, the mid/hindbrain boundary (MHB; also called the isthmus organizer) and the floor plate, a specialized cell type that lies along the CNS ventral midline. The secreted molecules of Shh and Fgf8 at the intersection give rise to DA neurons rostrally of the MHB and 5′HT neurons caudally of this boundary. In addition to Shh and Fgf8, 5′HT neurons require signaling by Fgf4, which might be derived from the primitive streak at earlier developmental stages.

Traditionally, the pineal and serotoninergic neurones are considers to be the seat of tryptophane metabolism with the expression of indoleamines. Tryptophane is converted to serotonin by MAO and further to melatonin by the light sensitive enzymes NAT and HIOMT during the dark phase. [28] It is observed that the highly dendritic melanocytes in the pigmented proliferative lesions and in melanomas express both serotonin and melatonin in the cytoplasm. [7] These cells show positivity for MAO with tryptamine as substrate. Thus, as in the case of the tyrosine metabolism the melanocytes show the entire machinery for indoleamine metabolism. The conversion of serotonin to melatonin is inhibited on exposure to a pulse of UV indicating the presence of the light sensitive enzymes NAT/HIOMT. [5]

The epidermal melanocytes metabolise tryptamine [29] to produce indoleamines on UV exposure in whole skin organ cultures. In the TVC, indoleamines are positive in L2/L3 associated with mitosis. Both serotonin and melatonin are positive in these layers. Conversion of serotonin to melatonin requires photosensitive enzymes HIOMT/NAT which are switched off by light. [28] A high mitotic activity in L2/L3 is a likely source of biophoton emissions of UV. [30] Since this activity is cyclical with light and dark phases, both SER & MLT are expressed in these layers. In turn, the indoleamines modulate the melanocyte cell cycle in response to UV exposure. [4-6]

Tyrosine is metabolised by several cell types which include catecholaminergic neurones in the brain and spinal cord, [31] sympathetic ganglia, adrenal medulla, and melanocytes which are neuroectodermal in origin. The metabolic pathway is through conversion of tyrosine to dopa, by catecholoxidase, a triphasic enzyme which catalyses both wings of dopa metabolism. [32]The catecholaminergic neurones of the locus ceruleus and substantia nigra produce both catecholamines as well as melanin. [33] This feature is observed in sympathetic ganglion cells [34] and occasionally in pigmented lesions of nerve sheath cells and Schwann cells all cells metabolising tyrosine. [35] The dendritic melanocytes in the skin, rich in enzyme positive melanosomes, show tyrosine hydroxylase and dopamine (DA) positivity, dopamineoxidase (DAO) activity and noradrenalin (NA) in response to UV exposure. [13] Thus the melanocyte has the enzyme machinery for the conversion of dopa to dopamine and dopamine to noradrenalin.

In the TVC, catecholamines DA/NA and the related enzyme DO are positive in the layers L3/L4 & L5. DO activity rises to 45% in L3 coinciding with initiation and peak DA positivity in L3/L4. Peak DO levels are seen in L4 coinciding with peak NA positivity, DO/Pig appearing in L4/L5 layers. In the TVC, DO peaks in L4 and pigment in L5, indicating a similar reciprocal relationship as seen in the skin [36] between DO levels in L4 and pigment in L5. In the skin catecholamines are expressed during S/G2 arrest on UV exposure. [10,11] As with indoleamines, the likely source of UV is the biophoton UV released in L2/L3 by the high mitotic activity with a resultant S/G2 arrest in the postmitotic cells in L4/L5. [30]

Thus there is a definite sequential pattern of differentiation of tumor cells interacting with angiogenic vessels in the TVC. The initial layers are positive for nestin and GFAP, the cells showing features of multipotent astrocytic stem cells. This is followed by the appearance of indoleamine positivity at L2/L3, the layers which show mitosis. Mitotic activity is insignificant in L4/L5, the layers positive for catecholamines, DO and pigment, suggesting that these are postmitotic cells. The results suggest that the mitosis in L2/L3 is asymmetric, one giving rise to indoleamine positive cells and

related enzymes, which remain in L2/L3, and a second to catecholamines positive cells and related enzymes, which move out into L4/L5 thus establishing a definite polarity (Figure 4e&f).

Cell polarity is essential for generating cell diversity and for the proper function of most differentiated cell types. Neuroblasts divide asymmetrically to result in apical/basal polarity in mitotic neuroblasts, to generate an apical neuroblast and a smaller basal ganglion mother cell (GMC) which undergoes one subsequent cell division to generate neurons or glia. [37-39]

Embryonic microenvironments influence development of neuronal stem cells. [40-43] Similarly multipotent tumor cells, fate and plasticity of adult cell are influenced by the microenvironment. [44-48] Multiple cell types which originate from the highly invasive neural crest cell population of the vertebrate embryo can be reprogrammed by simulating a natural environment in 3D organ culture. [40,44,49-54] Human metastatic melanoma cells respond to chick embryo NC-rich microenvironment. Highly aggressive human melanoma cells secrete Nodal, (a potent embryonic morphogen), to induce and form a secondary ectopic embryonic axis in zebrafish. [55-57]

As seen in the present study, during angiogenesis in aggressive melanomas, a microenvironment is created in the mantle zone of TVC around the vessel to induce and recapitulate early embryonal neurogenesis of indoleaminergic and catecholaminergic cells, much in the manner of the neural tube as summarized in fig.5. The angiogenic vessel confers a polarity to the interacting tumor cells which wrap themselves around. The aggressive melanoma cells function as neuronal progenitor stem cells within the narrow confines of the mantle zone, in response to angiogenesis. These features can be utilized to get a rich harvest of progenitor cells. These results indicate melanomas could be a rich source for harvesting indoleaminergic as well as catecholaminergic cells for therapeutic use on cocultures with endothelial cells using melanomas as stem cells.

Acknowledgement: I am indebted to:The Institute of Pathology, (ICMR), New Delhi for the technical support;Dr. KK Pandey, formerly the Head of Cancer Surgery, Safdarjung Hospital, New Delhi;Mr. Arvind Pundir, Technical Officer, National Brain Research Centre, Manesar, for nestin IHC staining.Dr. Soumya Iyengar, Associate Professor, National Brain Research Centre, Manesar, for the Statistical analysis.

REFERENCES

[1] Le Douarin, NM. The Neural Crest. Cambridge University Press;1982.

[2] Kalcheim, C, Le Douarin, N. The Neural Crest. Cambridge University Press; 1999.

[3] Sharma, K, Korade, Z, Frank, E. Late-migrating neuroepithelial cells from the spinal cord differentiate into sensory ganglion cells and melanocytes. Neuron 1995;**14**: 143-152. http://dx.doi.org/10.1016/0896-6273(95)90248-1

[4] Iyengar, B. Indoleamines and the UV-light sensitive photoperiodic responses of the melanocyte network: A biological calendar? Experientia 1994a; 50: 733-736. PMid:8070534 http://dx.doi.org/10.1007/BF01919373

[5] Iyengar, B. Photomodulation of the Melanocyte Cell cycle by Indoleamines. Biol Signals Recept 1998a; 7:345-350.

http://dx.doi.org/10.1159/000014558

[6] Iyengar, B. Role of serotonin in Melanocyte Functions. J Basic Appl Biomed 1998b; 6:11-14.

[7] Iyengar, B. The UV responsive melanocyte system: a peripheral network for photoperiodic time measurements - a function of indoleamine expression. Acta Anat 1998c; 163:173-178. http://dx.doi.org/10.1159/000046495

[8] Iyengar, B, Singh, AV. Patterns of neural differentiation in Melanomas. J Biomed Sci 2010; 17: 87.

PMid:21080952 PMCid:2997767 http://dx.doi.org/10.1186/1423-0127-17-87

[9] Lauder, JM, Bloom, FE. Ontogeny of monoamine neurons in the locus coeruleus, raphe nuclei and substantia nigra of the rat. I. Cell differentiation. J. Comp. Neurol 1974;155: 469–481.

[10] Iyengar, B. Neural differentiation as an expression of UV sensitivity of melanocytes. Acta Anat 1992a; 143:236-240.

http://dx.doi.org/10.1159/000147254

[11] Iyengar, B. Melanocytes - A UV sensitive neural network and Circadian Rhythms in man. Acta Anat 1992b; 144:332.

http://dx.doi.org/10.1159/000147326

[12] Luna, LG. Routine staining procedure. In: Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3rd Edition. 1968; 33-46.

[13] Pearse, AGE. Histochemistry theoretical and applied. Vol II: Analytical Technology. Churchill Livingstone, London. 1985; 611-674.

[14] Prophet, ED, Mills, B, Arrington, JB, Sobin, LH. Laboratory Methods in Histotechnology. American Registry of Pathology, Armed Forces Institute of Pathology, Washington DC. 1994.

[15] Mikel, UV. Advanced Laboratory Methods in Histology and Pathology . American Registry of Pathology, Armed Forces Institute of Pathology, Washington DC. 1994.

[16] Wiese C, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, et al. Nestin expression--a property of multi-lineage progenitor cells? Cell Mol Life Sci 2004;61:2510-2522. PMid:15526158 http://dx.doi.org/10.1007/s00018-004-4144-6

[17] Veselska, R, Kuglik, P**,** Cejpek, P**,** Svachova, H **,** Neradil, J **,** Loja, T, Relichova, J. Nestin expression in the cell lines derived from glioblastoma multiforme. BMC Cancer 2006: 6:32doi:10.1186/1471-2407-6-32.

PMid:16457706 PMCid:1403792 http://dx.doi.org/10.1186/1471-2407-6-32

[18] Ehrmann, J, Kolář, Z, Mokrý, J. Nestin as a diagnostic and prognostic marker: immuno histochemical analysis of its expression in different tumours. J Clin Pathol. 2005;58(2):222-223.

PMID:15677549 http://dx.doi.org/10.1136/jcp.2004.021238

[19] Messam, CA, Hou, J, Major, EO. Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody. *Exp Neurol.* 2000;161:585-96.

PMid:10686078 http://dx.doi.org/10.1006/exnr.1999.7319

[20] Suzuki, S, Namiki, J, Shibata, S, Mastuzaki, Y, Okano, H. The Neural Stem/Progenitor Cell Marker Nestin Is Expressed in Proliferative Endothelial Cells, but Not in Mature Vasculature. J Histochem Cytochem 2010;58:721–730.

PMID:20421592 http://dx.doi.org/10.1369/jhc.2010.955609

[21] Raju, T, Bignami, A, and Dahl, D. *In vivo* and *in vitro* differentiation of neurons and astrocytes in the rat embryo. Immunofluorescence study with neurofilament and glial filament antisera. *Dev Biol* 1981;85: 344-357.

http://dx.doi.org/10.1016/0012-1606(81)90266-9

[22] Zecevic, N. Specific characteristics of radial glia in the human fetal telencephalon. *GLIA* 2004;48:27-35.

PMid:15326612 http://dx.doi.org/10.1002/glia.20044

[23] Horst, H. Bhatt, SL, Gherbassi, D, Sgado, P, Alberi, L. Midbrain Dopaminergic Neurons Determination of Their Developmental Fate by Transcription Factors. Ann NY Acad Sci 2003;991: 36–47. PMID:12846972

[24] Wallen, A and Perlman, T. Transcriptional Control of Dopamine Neuron Development. Ann NY Acad Sci 2003**;** 991: 48–60**.**

http://dx.doi.org/10.1111/j.1749-6632.2003.tb07462.x

[25] Hynes, M and Rosenthal, A. Specification of dopaminergic and serotonergic neurons in the vertebrate CNS**.** Curr Opin Neurobiol 1999;9: 26–36.

http://dx.doi.org/10.1016/S0959-4388(99)80004-X

[26] Ye, W., et al**.** FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural tube**.** Cell 1998;93**:** 755–766.

http://dx.doi.org/10.1016/S0092-8674(00)81437-3

[27] Hobert, O. How to make dopamine neurons: *In:* ebrief: Neural Stem Cells: The New York Academy of Sciences ; March, 2008.

[28] Sugden, D. Melatonin biosynthesis in the mammalian pineal gland. Experientia 1989;45: 922-932.

PMid:2572451 http://dx.doi.org/10.1007/BF01953049

[29] Iyengar, B: Photoresponses of melanocytes. The circadian rhythm and proliferation. In: Iyengar B, Singh AV[eds] Growth Disorders of the Pigment Cells. BI Churchill Livingstone, New Delhi, 1996; 41-52.

[30] Iyengar, B: UV guided dendritic growth patterns and the neural networking of melanocytes. Experientia 1994b; 50: 669-672.

PMid:7913446 http://dx.doi.org/10.1007/BF01952870

[31] Graham, DG: On the origin and significance of neuromelanin. Arch Pathol Lab Med 1979;103:359-362.PMid:582279

[32] Nagatsu, T, Namata, SY, Kato, T et al. Effect of melanin on tyrosine hydroxylase and phenylalanine hydroxylase. Biochem Biophys Acta 1978;523:47-52, PMid:24474

[33] Miranda, M, Botti, D, Ventura, T, Bonfigli, A, Arcadi, A, Dicola, M. Melanogenesis in human substantia nigra. In.Bagnara J, Klaus SN, Schartl M[eds]. Pigment Cell, Tokyo, Univ. of Tokyo Press. 1985;31-36.

[34] Williams, PL, Warwick, R, Dyson, M, Bannister, LH[eds].Gray's Anatomy. 37th ed. Churchill Livingstone, London, 1989.

[35] Carpenter, MB: Core Textbook of Neuro anatomy. 3rd ed. Williams and Wilkins. Baltimore, USA, 1945.

[36] Rapini, RP, Bolognia, JL, Jorizzo, JL. Dermatology: 2-Volume Set. St. Louis: Mosby. 2007.

[37] Rolls, MM, Albertson, R, Shih, HP, et al.. Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. J Cell Biol 2003;163:1089-1098. PMid:14657233 PMCid:2173607 http://dx.doi.org/10.1083/jcb.200306079

[38] Doe, CQ. Molecular markers for identified neuroblasts and ganglion mother cells in the Drosophila central nervous system. Development. 1992;116:855-863.PMid:1295739

[39] Zhong, W. Diversifying neural cells through order of birth and asymmetry of division. Neuron 2003;37: 11-14.

http://dx.doi.org/10.1016/S0896-6273(02)01178-9

[40] Kulesa, JC, Teddy, JM, Postovit, LM, Seftor, EA, Seftor, RE, Hendrix, MJ, Kulesa, PM. Reprogramming multipotent tumor cells with the embryonic neural crest microenvironment Dev Dyn. 2008;237:2657-66.

PMid:18629870 PMCid:2570047 http://dx.doi.org/10.1002/dvdy.21613

[41] Hochedlinger, K, Blelloch, R, Brenna, C, Yamada, Y, Kim, M, Chin, L, Jaenisch, R. Reprogramming of a melanoma genome by nuclear transplantation. Genes Dev 2004;18:1875–1885. http://dx.doi.org/10.1101/gad.1213504

[42] Takahashi, K, Tanabe, K, Ohnuki, M, Narita, M, Ichisaka, T, Tomoda, K, Yamanaka, S. Induction of plutipotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872. PMid:18035408 http://dx.doi.org/10.1016/j.cell.2007.11.019

[43] Yu, J, Vodyanik, MA, Smuga-Otto, K, Antosiewicz-Bourget J, France, JL, Tian, S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917–1920. PMid:18029452 http://dx.doi.org/10.1126/science.1151526

[44] Hendrix, MJ, Seftor, EA, Seftor, RE, Kasemeier-Kulesa, JC, Kulesa, PM, Postovit, LM. Reprogramming metastatic tumour cells with embryonic microenvironments. Nat Rev Cancer. 2007;4:246–255. PMid:17384580 http://dx.doi.org/10.1038/nrc2108

[45] Real, C, Glavieux-Paranaud, C, LeDouarin, NM, Dupin, E. Clonally cultured differentiated pigment cells can dedifferentiate and generate multipotent progenitors with self-renewal potential. Dev Biol. 2006;300:656–669.

PMid:17052705 http://dx.doi.org/10.1016/j.ydbio.2006.09.032

[46] Graham, A, Begbie, J, McGonnell, I. Significance of the cranial neural crest. Dev Dyn. 2004;229:5–13.

PMid:14699573 http://dx.doi.org/10.1002/dvdy.10442

[47] Kulesa, P, Ellies, DL, Trainor, PA. Comparative analysis of neural crest cell death, migration, and function during vertebrate embryogenesis. Dev Dyn 2004;229:14–29.

PMid:14699574 http://dx.doi.org/10.1002/dvdy.10485

[48] Harris, ML, Erickson, CA. Lineage specification in neural crest cell pathfinding. Dev Dyn 2007;236:1–19.

PMid:16894594 http://dx.doi.org/10.1002/dvdy.20919

[49] Le Douarin, NM, Dupin, E. Multipotentiality of the neural crest. Curr Opin Genet Dev 2003;13:529–536.

http://dx.doi.org/10.1016/j.gde.2003.08.002

[50] Le Douarin, NM, Creuzet, S, Couly, G, Dupin, E. Neural crest cell plasticity and its limits. Dev 2004;131:4637–4650.

PMid:15358668 http://dx.doi.org/10.1242/dev.01350

[51] Bronner-Fraser, M, Fraser, SE. Cell lineage analysis reveals multipotency of some avian neural crest cells. Nature 1988;335:161–164. PMid:2457813 http://dx.doi.org/10.1038/335161a0

[52] Tickle, C, Trinkaus, JP. Observations on nudging cells in culture. Nature 1976;261:413.

PMid:934272 http://dx.doi.org/10.1038/261413a0

[53] Bilozur, ME, Hay, ED. Neural crest migration in 3D extracellular matrix utilizes laminin, fibronectin, or collagen. Dev Biol 1988;125:19–33.

http://dx.doi.org/10.1016/0012-1606(88)90055-3

[54] Hay, ED. The extracellular matrix in development and regeneration. An interview with Elizabeth D. Hay. Int J Dev Biol 2004;48:687–694. PMid:15558460

http://dx.doi.org/10.1387/ijdb.041857rt

[55] Mintz, B, Illmensee, K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc Natl Acad Sci 1975;72:3585–3589. http://dx.doi.org/10.1073/pnas.72.9.3585

[56] Topczewska, JM, Postovit, LM, Margaryan, NV, Sam, A, Hess, AR, Wheaton, WW, et al. Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. Nat Med 2006;12:925–932.

PMid:16892036 http://dx.doi.org/10.1038/nm1448

[57] Kulesa, PM, Kasemeier-Kulesa, JC, Teddy, JM, Margaryan, NV, Seftor, EA, Seftor, RE, Hendrix, MJ. Reprogramming metastatic melanoma cells to assume a neural crest-cell like phenotype in an embryonic microenvironment. PNAS 2006;103:3752–3757. PMid:16505384 PMCid:1450149 http://dx.doi.org/10.1073/pnas.0506977103