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STROMAL RESPONSES IN HUMAN PRIMARY MELANOMA OF THE SKIN

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1. ABSTRACT

Tumour development and progression has long been considered as the consequence of an imbalance between apoptosis and proliferation of transformed cells. However, whereas genetic aberrations leading to the activation of oncogenes and/or loss of tumour suppressor genes are crucial for the transformation towards aberrant cell growth, progression towards a full blown malignancy requires a dynamic interaction between tumour cells and the environment in which they thrive. Over the recent years, it has become evident that the (early) inflammatory and angiogenic response, and remodelling of the extracellular proteins are key factors in creating a microenvironment that sustains tumour growth and metastasis. The host response towards cutaneous melanoma has received relatively little attention, most likely because the majority of these tumours develop without evoking a strong stromal response as can be observed in, *e.g.*, carcinomas. This review discusses potential critical modulators of melanoma growth: turn-over of the most abundant extracellular matrix protein in skin (*i.e.* type I collagen), the early inflammatory response and angiogenesis

2. INTRODUCTION

Human cutaneous melanoma frequently develops in a sequence of steps from benign proliferative nevi to atypical nevi, non-invasive in-situ melanomas, invasive melanomas and finally to metastases (1). Clark (2) depicted a stage-wise progression of the relatively indolent radial growth phase (RGP) to the deeply invasive vertical growth phase (VGP) with metastasizing capacity (3). Melanoma appears to progress through these different stages of cumulative malignancy without evoking a strong host response towards the developing neoplasm. However, subtle changes in extracellular matrix protein turn-over, infiltration of early inflammatory cells, and an angiogenic response can be observed.

3. CUTANEOUS MELANOMA AND TYPE I COLLAGEN TURN-OVER

The two growth phases that can be identified in primary cutaneous melanoma are each embedded with a unique extracellular matrix. RGP and early VGP melanomas are confined to the upper papillary dermis, whereas advanced VGP melanomas invade the reticular

dermis. Dermal connective tissue is mainly composed of collagens which not only maintain the structural integrity of skin, but also regulate cell polarity, migration, survival and phenotype. Within the family of collagens, type I collagen is included in the group of fibril-forming collagens based on its structural and functional features. It accounts for 80-90% of all collagenous proteins in skin (4) and is composed of one alpha2 and two alpha1 chains in a triple helix conformation. Type I collagen is synthesized as a soluble procollagen (5) and is assembled into collagen fibrils and fibres after the globular C- and N-propeptides are cleaved off. In the papillary dermis, type I collagen is found as a finely woven meshwork of fibres, whereas it is assembled into thick bundles in the reticular dermis (5). With the exception of the desmoplastic subtype, melanoma does not evoke a strong stromal response with respect to matrix protein synthesis by tumour-associated fibroblasts as is observed in cutaneous squamous cell carcinoma (6). However, type I collagen mRNA in situ hybridization revealed *de novo* expression by fibroblasts around nests of micro-invasive melanoma cells in the papillary dermis, but not at the edge of deeply invasive, advanced VGP melanoma cells (Figure 1).

We have reported previously that melanoma progression coincides with increased expression of various matrix protein degrading enzymes (e.g. matrix metalloproteinase (MMP)-1, 2, 8 and 9) in deeply invasive melanomas (7,8,9). Increased expression of collagen degrading enzymes by tumour and stromal cells contributes to destabilisation of the type I collagen matrix. AZAN-staining of human melanoma lesions indeed revealed a loosely woven collagen architecture around small nests of melanoma cells deeply invading the dermis, suggestive for pericellular remodelling of the ECM (Figure 1). In summary, RGP and early VGP melanomas are embedded within a collagen matrix that is not subjected to degradation and in which fibroblasts express type I collagen. This contrasts with pericellular matrix proteolysis and lack of collagen synthesis in the advanced VGP microenvironment.

In addition to maintaining the structural integrity, regulation of cell polarity, migration, survival and phenotype, type I collagen provides diffusible signalling molecules following breakdown. The conformation of type I collagen determines the binding affinity and, thus, bioavailability of various molecules (10). Until now, about 50 different molecules have been found to interact with type I collagen (11). Decorin and SPARC are non-collagenous matrix proteins ubiquitously expressed in connective tissue (12) that simultaneously interact with fibrillar collagen and growth factors (*e.g*, transforming growth factor (TGF)-beta1 (13), platelet-derived growth factor (PDGF) (14), fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) (15)). Upon binding to decorin or SPARC these growth factors become matrix-sequestered and unavailable for activating cellular signalling pathways, but can be released in a biologically active form by collagenase treatment (16).

In summary, detailed analysis of type I collagen synthesis and degradation in human melanoma revealed an altered tumour microenvironment of deeply invasive and metastatic melanoma in the reticular dermis, compared to micro-invasive melanoma confined to the papillary dermis. Although it remains unclear whether advanced VGP melanoma is a cause or consequence of the altered microenvironment, these data strongly suggest that the tumour microenvironment and melanoma-stroma interactions are critical determinants of neoplastic progression (17,18,19,20).

4. TYPE I COLLAGEN AND GROWTH INHIBITION OF MELANOCYTIC CELLS

Dermal connective proteins exert a growth inhibitory effect on cells whose growth is normally confined to the epidermis. Growth of melanocytes is normally restricted to the epidermis (21). Transformed melanocytes, which have invaded the dermis, however, have gained the ability to counteract apoptosis and grow in this new environment (22). Several mechanisms of apoptosis evasion and promotion of invasive growth can be exploited. FGF-2, is uniformly expressed by melanoma cells but not by normal melanocytes (23), and counteracts type I collagen-induced apoptosis via stimulating proliferation in an autocrine manner (24). Furthermore, *de novo* expression of the alpha(v)beta(3) vitronectin receptor by melanoma cells results in anti-apoptotic signalling and cell survival after binding of this receptor to type I collagen (25). Evasion of type I collagen-induced growth arrest and apoptosis can be achieved by collagen digestion. The transition from micro-invasive to deeply invasive melanoma coincides with increased expression of matrix metalloproteinases (MMPs) with collagenolytic activity, *i.e.*, MMP-1, -8, -13 and -14 ((26) and our unpublished results). Whereas melanoma cells cultured in fibrillar type I collagen are inhibited in their growth via activation of the receptor tyrosine kinase discoidin domain receptor (DDR)-2 (27), cell cycle arrest is abrogated when cells are grown in denatured collagen (28) and in which the DDR-2 receptor is no longer activated. Interestingly, only membrane anchored MMP-14 confers a growth advantage *in vivo* by stimulating cell proliferation, which strictly depends on the hydrolysis of the surrounding type I collagen matrix (29). Other collagenases may thus support neoplastic growth by liberating matrix-sequestered polypeptide growth factors (10).

5. TYPE I COLLAGEN TURN-OVER AND TUMOUR PROGRESSION

In general, tumour development and subsequent invasion into adjacent tissue is characterized by increased architectural disorder especially at the invasive front of the neoplastic mass (30). Here, along with increased production of proteinases (31,32,33), increased synthesis of many matrix components has been documented. Most notably, fibroblasts migrate towards the hyperproliferative tissue where they synthesize new matrix components. In skin, two different host responses with respect to type I collagen expression by tumour-associated fibroblasts can

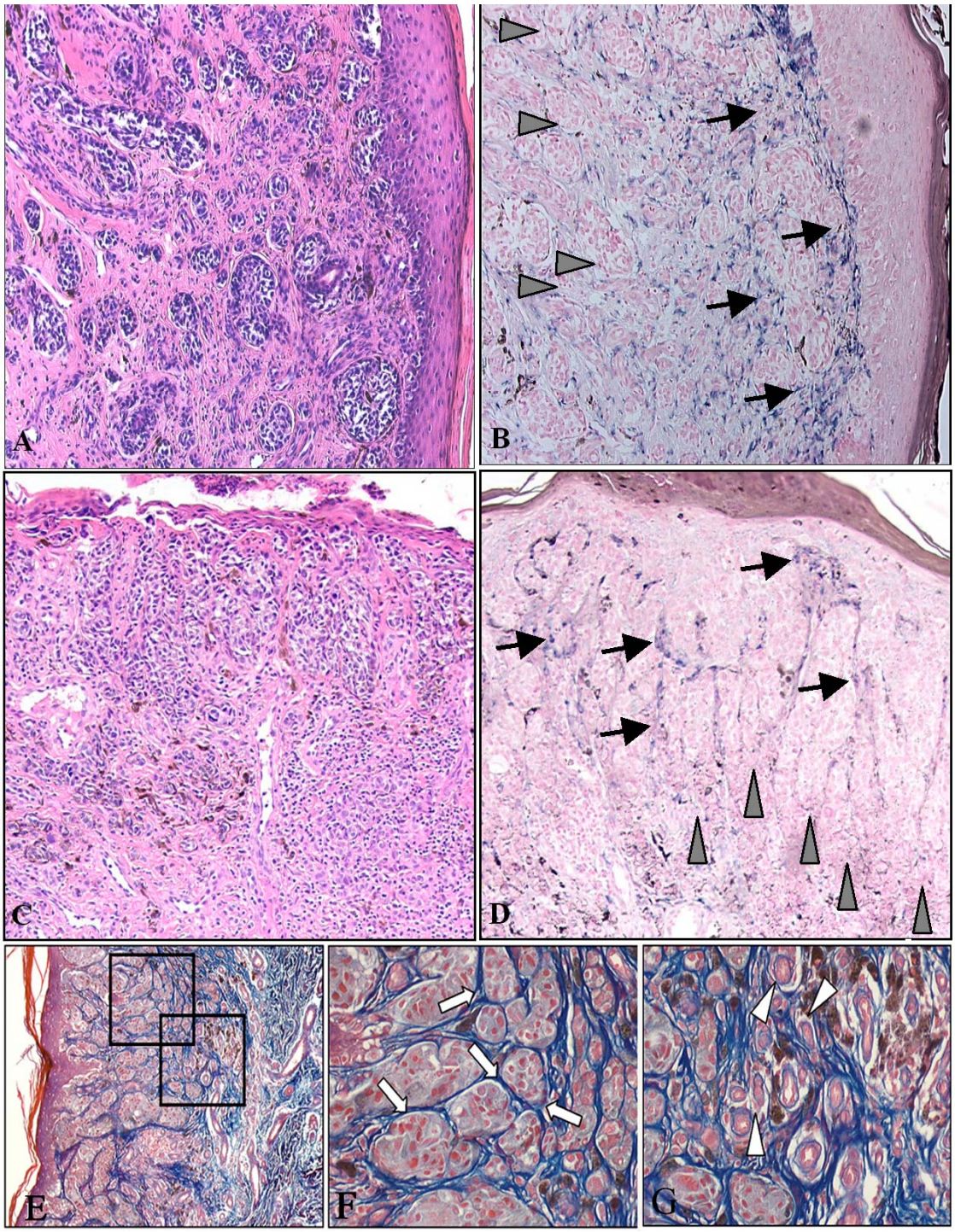

Figure 1. Type I collagen turn-over in human melanoma. *Type I collagen expression.* Type I collagen is expressed by fibroblasts juxtaposed to melanoma cells in the superficial component of the tumour but not by fibroblasts adjacent to deeply invasive nests of melanoma cells. In situ hybridization of type I collagen alpha 1 mRNA on paraffin-embedded melanoma lesions revealed type I collagen expression by fibroblasts in the superficial component of melanomas (B and D, arrows). In contrast, type I collagen a1 mRNA could not be detected in the stroma of the deeply invasive tumour component (B and D, arrowheads). (A and C, H&E staining of a parallel tissue section). Precursor stages of melanoma (atypical nevi) did not evidence type I collagen expression (not shown). *Collagen remodelling*. AZAN staining of human melanoma (E) reveals a tightly woven collagen network (arrows, F) in the papillary component of the tumour. In contrast, whereas loss of deeply invasive nests of melanoma cells (arrowheads, G) is indicative of local matrix remodelling.

be observed. Whereas *de novo* expression of type I collagen is only detectable in RGP melanoma and pericellular degradation in VGP melanoma (Figure 1), murine and human cutaneous squamous cell carcinoma (34,35) display a simultaneous increase in type I collagen synthesis and degradation when the tumour expands into the papillary dermis. Type I collagen synthesis by fibroblasts can be induced by a variety of factors produced by melanoma and/or stromal cells *e.g* TGF-beta1, PDGF, IL-1alpha and -beta, and mast cell derived IL-4 and tryptase (reviewed in (20)). In contrast, melanoma cell-derived FGF-2 (24) and IL-10 (36), and T-cell-derived interferon-gamma can inhibit type I collagen synthesis in a concentration dependent manner *in vitro* (37,38,39,40). In addition, the simultaneous secretion of proteinases by tumour and host cells (see 7.2) can degrade newly synthesized and pre-existing collagen fibres. These data indicate that the balance between type I collagen degrading, and synthesis stimulating and inhibiting factors determines collagen turn-over and that this not only depends on tumour type, but also on the stage of progression.

6. TYPE I COLLAGEN INTERACTING MOLECULES

The architecture of the extracellular matrix determines the local bio-availability of collagen-binding peptides and binding affinity for a variety of collagen receptors. Until now, about 50 molecules have been found to interact with type I collagen and for about half of them, binding sites on this collagen have been elucidated (11). Availability of these binding sites can depend on the conformation of type I collagen. For example, collagenase-mediated cleavage of type I collagen affects binding of alpha(1)beta(1) and alpha(2)beta(1) integrins to type I collagen (41,42). Similarly, only fibrillar collagen can activate DDR1 and DDR2 (43,44) Activation of DDR2 expressed by fibroblasts induces MMP-1 and MMP-2 expression (45,44), whereas activation of DDR1 expressed by tumour cells (including cutaneous melanoma, our unpublished data) results in growth inhibition (46,47). Collagenase-cleaved collagen, however, does not activate the DDR receptors (43,44), demonstrating that the nature of the ECM can define cellular shape and behaviour (48).

The conformation of type I collagen can also indirectly determine the bio-availability of soluble signalling molecules. Secreted protein acidic and rich in cysteine (SPARC) and decorin are a non-collagenous matrix proteins ubiquitously expressed in connective tissue (15,49,12). Because of their ability to interact with collagen and growth factors, *e.g,* TGF-beta1 (13), PDGF (14), FGF-2, VEGF, and EGF (15), they have been implicated in the control of matrix assembly and cellular growth. Upon binding to SPARC or decorin, these growth factors become matrix-sequestered and unavailable for activating cellular signalling pathways. However, they can be released in a biologically active form by collagenase treatment (16). In summary, type I collagen-binding molecules and type I collagen conformation can determine the bio-availability and/or activity of growth factors and collagen receptors, thereby modulating various cell signalling pathways involved in growth and behaviour of all cells within a tumour.

7. EARLY INFLAMMATORY RESPONSES, ANGIOGENESIS AND MATRIX REMODELLING

Although tumour cells can secrete growth factors that can stimulate fibroblasts to produce type I collagen, inflammatory cells play an equally important role in creating a tumour stroma to facilitate growth. Collagen turn-over not only modulates the local bio-availability of collagen-binding growth factors; concerted synthesis and degradation of type I collagen facilitates angiogenesis.

7.1. Inflammation and angiogenesis

Development of cutaneous melanoma is characterized by infiltration of inflammatory cells early in the onset and throughout tumour development, and involves recruitment of mast cells and monocytes/macrophages. Mast cells are the first cells that migrate to sites of proliferation in response to a variety of (tumour-derived) polypeptide growth factors, which include stem cell factor (SCF), VEGF, EGF, FGF-2, PDGF (reviewed in (50)). The neuropeptide substance P that can be secreted by both dermal neurons, nevus and melanoma cells (51) results in degranulation of mast cells at these sites and in turn results in the release of histamine (52), vesicle-stored VEGF (53), several serine proteases including chymase and tryptase (54) and matrix MMP-9 (53,55). In addition to the presumed matrix remodelling activity of MMP-9 (reviewed in (10)), this proteinase is known to mediate release of matrix-sequestered VEGF (56), thereby enhancing angiogenic responses that further facilitate the initial burst of tumour growth. In a transgenic mouse model for SCC development, bone marrow-derived inflammatory cells are the main sources of MMP-9 during tumour development (57) and infiltration of mast cells appears to induce activation of the angiogenic switch (58). These data suggest that mast cell-derived MMP-9 (59) is a critical mediator involved in inducing an angiogenic response in carcinomas (57,30,60). In melanoma, increased microvessel density is observed in the thick and vertical growth phase melanomas, but not in (micro-invasive) RGP (61,62). The observations that (i) MMP-9 is variably expressed in (micro-invasive) radial growth phase melanoma (63), (ii) melanoma *in situ* evidences increased density of lesional mast cells compared to nevi (64), and that (iii) peritumoural accumulation of mast cells correlates with microvessel density and poor prognosis in melanoma (65,64) suggests mast cells accumulation and release of MMP-9 precedes the angiogenic response facilitating the transition of RGP to VGP melanoma.

During tumour development, mast cell infiltration is often followed by macrophages recruitment (reviewed in (66)). Macrophages are a major source of growth factors and cytokines that profoundly affect endothelial, epithelial and mesenchymal cells in the local microenvironment (66). Macrophages are differentiated monocytes that originate from bone marrow and differentiate upon extravasation

from the blood circulation. They are recruited to sites of tissue injury, inflammation or proliferation by specific chemokines, *e.g.* monocyte chemotactic protein (MCP)-1 (67) as well as various polypeptide growth factors (68). Following recruitment, macrophages respond to micro-environmental factors by producing important mitogens as well as various growth factors and enzymes that stimulate angiogenesis (69,70) - a prerequisite for tumour growth (71). Once "recruited", tumour-associated macrophages (TAMs) can be activated via tumour-derived TGF-beta1 and respond by secreting TNF-alpha, IL-1, -6, and pro-angiogenic IL-8 or bFGF (72,73). Reciprocally, neoplastic cells secrete increased levels of IL-8, FGF-2 and VEGF (74,75), thereby further enhancing angiogenic responses. TAM-induced vascular responses can be attenuated by IL-10 that efficiently squelches synthesis of TAM-derived VEGF, IL-1β, TNF-α, IL-6 and MMP-9 (76). Taken together, these data suggest an important role for macrophages in the induction of tumour-associated angiogenic responses.

Infiltration of macrophages in melanoma precursor lesions and association with angiogenesis and melanoma progression is counterintuitive. In tumours, macrophages may aid in invasion of neoplastic cells into surrounding stroma (77) and direct remodelling of stroma rendering it suitable for appropriate angiogenic and/or lymphangiogenic responses (78,79). The biologic effect of TAMs, however, depends on the level of tumour-derived MCP-1 secretion and overall level of macrophage recruitment (74). Studies with MCP-1 expressing tumour cells suggest that low concentrations of MCP-1 results in a modest level of macrophage recruitment and enhances angiogenesis and tumour growth in a melanoma xenograft mouse model (74). In contrast, a massive infiltration of macrophages in tumours due to high levels of MCP-1 results in increased vascularisation and tumour growth, but eventually also in tumour regression. In addition, data suggest that tumour cells can divert anti-tumour macrophage responses and suppress differentiation of mature tumour-antigen-presenting dendritic cells, thereby evading the host immune response (reviewed in (77)). Taken together, these data suggest that anti-tumour macrophage responses depend on the balance between the number of infiltrating macrophages and the inhibition of their anti-tumour activity. A relatively low level of macrophage infiltration results in promoting neoplastic progression by increasing the level of angiogenic factors via direct or indirect mechanisms.

7.2. Inflammation and matrix remodelling

New blood vessel formation induced by angiogenic molecules during wound healing or tumour growth not only requires vessel sprouting from pre-existing vasculature, but also remodelling of ECM enabling embedding of endothelial cell tubes and associated pericytes into perivascular stroma (80,81,82,83). This process is characterized by the activation of multiple protease cascades, including MMPs, matrix remodelling and a simultaneous induction in the synthesis of key ECM molecules, *e.g.* collagens, laminin (84). Inflammatory cells are recruited to (pre)malignant lesions or sites of tissue injury where they affect local tissue architecture by releasing and/or stimulating production of ECM remodelling enzymes of the matrixin gene family (reviewed in (85)) as well as other proteolytic enzymes, *e.g.* urokinase plasminogen activator (reviewed in (86)). Whereas degranulation of mast cells, and macrophages results in a direct release of granule-stored MMP-9 (58,55,59), MMP-12 (87) and MMP-8 (88), inflammatory cells simultaneously release cytokines (*e.g* IL-1β, TNFα) that also have a modulating effect on MMP-synthesis by fibroblasts (reviewed in (89)).

Multiple cell types in neoplastic microenvironments secrete MMPs in response to paracrine signalling induced by interleukins, interferons, EMMPRIN, polypeptide growth factors and chemokines (90). Whereas melanoma cells can synthesize MMPs, they are predominantly produced by stromal cells at specific stages of tumourigenesis (91,26,92). Matrix degradation by MMPs has long been viewed as essential for tumour progression since focal remodelling of ECM barriers theoretically permits invasion of malignant cells into the surrounding tissue, entry and exit from venous and lymphatic vasculature, cumulating in metastases (reviewed in (10,85)). Moreover, MMPs participate in the release of matrix sequestered angiogenic and mitogenic factors that affects both tumour cells and cells in the microenvironment (93,10). In squamous cell carcinoma, inhibition of MMP activity using synthetic inhibitors reduced inflammation-induced epidermal hyperplasia in murine skin (94), suggesting that the inflammatory response and associated MMP activity is a critical parameter of early stage squamous carcinogenesis. In sum, the data suggest that infiltration of macrophages and mast cells in early stage tumours and/or their premalignant precursors (epidermal hyperplasia and melanoma *in situ*), may be of critical importance for initiating, and sustaining pre-malignant growth by generating a microenvironment that favours further neoplastic progression.

8. MELANOBALSTOMA-BEARING LIBECHOV MINIPIGS, A MODEL FOR CUTANEOUS MELANOMA

Various animal models are used to study melanoma progression (95,96,97,98). In order to study the role of the ECM on early stages of melanoma progression, mouse models are not preferred because murine melanomas arise from dermal or hair follicle-associated melanocytes, which is in sharp contrast with human melanocytic lesions that arise from the epidermis. Swine skin presents morphological and functional characteristics comparable to human skin. The ultrastructure of the epidermal-dermal junction, enzyme pattern of the epidermis, epidermal tissue turn-over time, keratinous proteins, cellular composition and thickness of the epidermis of porcine and human skin are similar (99,100). Furthermore, wound healing in swine skin is similar to that in human skin (101). Therefore, swine appears to be the most suitable experimental model for research related to human cutaneous malignancies. Swine from the melanoblastoma-bearing Libechov minipig (MeLiM) strain (95) are genetically predisposed to

cutaneous melanoma and provide a good opportunity to dissect the role of the microenvironment in human melanoma progression. Porcine melanocytic lesions arise from epidermal melanocytes and progress towards dysplastic nevi / melanoma i*n situ*, superficial spreading melanoma and nodular melanoma in a 1:1:1 ratio (102,99). Most of the pigmented cutaneous lesions appear *in utero* or in the first sixty days after birth, are distributed on various parts of body and mainly metastasize to lymph nodes and lungs within two to three months after manifestation. Genetic analysis of the MeLiM swine suggests an association of the swine 1q25 chromosomal region with melanoma susceptibility. This region is homologous to the human 9p21 region and is associated with a subset of human familial melanoma (103). In analogy to human melanoma, MeLiM melanoma progression exhibits an opposite regulation of tenascin-C and tenascin-X expression (104,105), thereby further strengthening the homology between swine and human melanoma. In contrast to human melanoma, however, a high degree of non-immunologic melanoma regression and associated melanosis is observed in lesions older than 4 months (106,99). Furthermore, porcine melanocytic lesions do not evidence a strong inflammatory cell response (99). In turn, this makes it easier to study tumour cell-fibroblast interactions as inflammation can result in fibroblast activation and type I collagen synthesis (20). To our opinion, MeLiM could be an excellent model to study the effect of collagen-turn on melanoma progression, providing that (i) the model reflects the spatial and temporal type I collagen expression profile as is observed in human melanoma and that (ii) adequate strategies can be developed to inhibit its expression using, *e.g.*, halofuginone (107,108), colchicines (109), phenytoin, nifedipine (110) or perfenidone (111), or lentivirus-mediated siRNA targeted to either the alpha(1) or alpha(2) chain of type I collagen.

9. GENERAL CONCLUSION

Human melanoma progression from RGP to VGP growth coincides with a subtle, but important alteration in type I collagen turn-over. The conformation of collagen has a strong effect on the local bio-availability of a wide variety of growth factors. Increased synthesis or pericellular proteolysis of type I collagen modulates the microenvironment, thereby potentially affecting all cells within or in close proximity of the tumour. Currently, experiments to reveal the contribution of collagen turn-over to melanoma development and progression are in progress and are part of our ongoing efforts to unravel the dynamic cross-talk between tumour cells, stromal cells and extracellular matrix proteins that is facilitating melanoma growth.

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