

Recent developments of 3D models of the tumor microenvironment for cutaneous melanoma: Bridging the gap between the bench and the bedside?

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Abstract

In cancer drug discovery, 3D approaches have been implemented over the last years to model the complex tumor architecture and the heterogenous components of the tumor microenvironment. Cutaneous melanoma represents a cancer of substantial unmet need with steadily growing incidence. Bioengineering techniques are increasingly applied to design melanoma-specific models. Here, we summarize how extracellular and cellular elements of the melanoma tumor microenvironment have been studied in 3D cell culture models. We review the benefits of such models and address their limitations and future perspectives for the development of novel therapeutic strategies.

Introduction

According to the world health organization (WHO) cancer is the second leading cause of death worldwide [1]. Although comprising <5% of all skin cancer cases, melanoma represents the most aggressive subtype accounting for >80% of skin-cancer related deaths and the numbers of cases are rising rapidly [2]. Accordingly, mortality and morbidity is relatively high with approximately 50.000 deaths annually worldwide, especially due to the occurrence of metastases [3]. After lung and breast cancer, melanoma is the third most common type of cancer likely to metastasize predominantly to the brain [4-6]. In fact, an estimated 10-40% of melanoma patients will develop brain metastases [7]. Thus, prognosis of patients with melanoma brain metastases (MBM) is poor, with an expected overall survival (OS) of only 4 months, [8-10].

In melanoma, the mitogen-activated protein kinase (MAPK) pathway is ubiquitously activated due to point mutations in the v-Raf murine sarcoma viral oncogene homolog B (BRAF) (35%-50% of melanomas) and in the Neuroblastoma RAS viral oncogene homolog (NRAS) (10%-25%). Efforts to target this pathway resulted in the approval of the BRAF inhibitors vemurafenib and dabrafenib, the mitogen-activated protein kinase (MEK) inhibitor trametinib, and the combination treatment of dabrafenib + trametinib [11]. Conventional therapy for MBM consists of whole brain radiation therapy for multiple metastases and radiosurgery or radiotherapy for limited numbers of metastases [12]. Despite the invention of new systemic drugs, such as immunotherapy with checkpoint inhibitors like anti-cytotoxic T-lymphocyte-associated protein 4 (ipilimumab) [13,14], anti-programmed cell death protein 1 (anti-PD1) (nivolumab and pembrolizumab) [15,16] or a combination [17], and targeted therapy (BRAF, MEK inhibitors) [18-21], outcomes remain poor and new efficacious therapies are urgently needed.

However, to date, the success rate of novel antitumor drugs transitioning in the clinic to phase 3 is in general relatively low [22-24]. Since clinical trials are, amongst others, initiated based on evidence of

efficacy in preclinical cell-based and mostly rodent models, clearly these models need to become more predictive in future drug development. The cellular heterogeneity of the tumor and its surrounding microenvironment combined with mechanical forces due to the three-dimensional (3D) architecture as well as hypoxia and gradients of nutrients may influence the efficacy of potential therapeutics, making it critical to consider 3D models when examining novel anti-cancer drug candidates.

This review aimed to describe the current concepts of 3D *in vitro* cutaneous melanoma models, compare the key characteristics of these 3D models versus two-dimensional (2D) models, and discuss the potential as well as the challenges of current 3D melanoma models for future drug discovery efforts.

Main part

The tumor microenvironment: A key player in melanoma carcinogenesis

The oversimplification of human tumors using 2D *in vitro* models explains why these “monolayer” models fail to capture the extent and nature of cell-cell and cell-extracellular matrix (ECM) interactions as well as nutrients and oxygen gradients that can occur due to the 3D configuration of human tumors [25]. Over the past decades, cancer research has been initially focused on cancer cells and on understanding their transformation into tumor cells. However, recently it has become clear that the tumor microenvironment (TME) with its cellular and molecular heterogeneity has a major impact on tumor progression as well as metastasis formation and spreading into distant tissues.

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Melanoma results originally from malignant transformation of melanocytes, which are naturally occurring pigmented cells in the epidermis to protect mitotically active keratinocytes against damage caused by UV-light irradiation. The melanocytes originate in the neural crest and consequently migrate to the epidermis. Melanocyte migration is guided by an intercellular crosstalk with i.e. keratinocytes [26]. Keratinocytes are also involved in melanoma progression [27] and their presence is essential to study cellular responses to drug candidates in a complete tumor microenvironment. Hence, the microenvironment of melanoma is formed not only by malignant cells but also by complex interactions with non-cancerous stromal cells, such as keratinocytes, cancer associated fibroblasts (CAFs), infiltrating immune cells, blood/lymphatic endothelial cells as well as biochemical components of the extracellular matrix, that all participate in melanoma formation and growth [28].

In addition to the highly heterogeneous cancer cell populations, the complex stromal tissue acts as a repository for various growth factors and cytokines that can greatly affect tumor growth and drug response [29-31]. Various growth factors secreted by CAFs also enhance an epithelial-mesenchymal transition (EMT) and the therapeutic resistance of cancer [32]. CAFs from melanoma influence also keratinocytes and, among others, induce expression of vimentin, a cytoskeletal protein involved in EMT, which is only weakly expressed in normal human keratinocytes [33]. Furthermore, 3D co-culture experiments showed that CAFs promote migration and invasiveness of melanoma cells and such migration is for instance dependent on interleukin-6 (IL-6) and interleukin-8 (IL-8) secretion [34]. Angiogenesis and metastases may be accompanied by secretion of IL-8 from tumor stroma confirming its prognostic significance as a circulating biomarker of melanoma [35]. Accordingly, serum levels of IL-8 correlate with tumor stage [36]. Similar to IL-8, production of hepatocyte growth factor (HGF) by stromal cells enhances melanoma invasiveness and elevated HGF levels in melanoma are accompanied by chemotherapy resistance [37]. Noteworthy, a variety of additional extracellular components including enzymes, such as matrix remodeling proteases, extracellular vesicles (EVs), such as exosomes [38], as well as EV transferred cargo including miRNAs [39], and nutrient and oxygen gradients synergistically participate in control of melanoma progression.

Hence, non-cancerous cells of the tumor microenvironment can significantly affect the efficacy of melanoma cells to therapeutic agents. For instance, in a vascularized 3D spheroid model melanoma cell proliferation after chronic treatment with vemurafenib was less pronounced in spheroid regions with fibroblasts, indicating an effect of the microenvironment on drug response [40]. Consequently, therapeutic manipulation of the tumor microenvironment has to be considered a highly promising approach which cannot be technologically addressed in classical 2D cell culture models.

The immune response in melanoma: Two sides of a medal

Improved understanding of the tumor microenvironment has resulted in innovative treatment approaches targeting the immune response, such as immunotherapy with checkpoint inhibitors like anti-cytotoxic T-lymphocyte-associated protein 4 (ipilimumab) [13,14] or anti-programmed cell death protein 1 (anti-PD1) (nivolumab and pembrolizumab) [15,16]. Among the immune cells infiltrating melanoma, the T-lymphocytes play a central role in anti-cancer immunity and, the degree of T-cell accumulation within the tumor is an important predictor of response of patients to immunotherapy [41]. Some common tumor microenvironment characteristics in melanoma

include the presence of tumor-associated macrophages of the M2-type (M2 TAMs) and regulatory T-lymphocytes (Treg) as non-tumor cells, which represent targets for immunotherapy by contributing to the escape of melanoma cells from immune defence mechanisms [42,43]. Distinct chemokines produced by tumor infiltrating macrophages may help to recruit Tregs to the tumor and maintain an immunosuppressive tumor microenvironment in melanoma [43]. As both cell types have a prominent immunosuppressive action, they promote skin carcinogenesis [44,45]. Notably, tumor infiltration by such immunosuppressive cells relates to unfavorable prognosis.

In contrast, other immune cells present in the tumor stroma, such as natural killer (NK) cells, M1-type macrophages or dendritic cells, have been reported to induce a protective anti-tumor immune response [46,47], underlying the ambiguous role of the immune response in melanoma. For instance, NK cells have an important role in innate and adaptive immunity through cytokine secretion and interaction with dendritic cells.

In conclusion, as immune cell components of malignant melanoma are essential for investigating novel treatment approaches, an integration into preclinical *in vitro* models appears mandatory.

Recent development in human 3D melanoma models

Like most cancer types, melanoma has been initially studied *in vitro* in conventional 2D cell culture models. Indeed, 2D cell culture has its benefits in low costs and as a fast method for high throughput drug screening with high reproducibility [48,49]. However, while 2D monolayer cell cultures have unlimited access to nutrients, oxygen, metabolites and signal molecules, 3D models may come closer to the physiological situation *in vivo* [48-50], as already indicated. In addition, when compared 2D and 3D cancer models for the same tumor entity, differences in cell metabolism, proliferation, apoptosis-related genes, chemoresistance related genes, the amount of cancer stemness, and miRNA profiles have subsequently been noted including melanoma [48,49,51].

In particular, for melanocytes and melanoma cells genetic alterations and loss of phenotype during 2D monoculture have been described. Already in 1987 it was observed that 2D culture conditions supporting the rapidly proliferation of human melanocytes, resulted in antigenic changes (expression of surface antigens as nerve growth factor receptor, proteoglycan, transferrin-related M, 120,000 protein etc.), associated with malignant melanomas [52]. Furthermore, mutations in the p16/CDKN2 tumor suppressor gene have been revealed in different melanoma cell lines during 2D culture, but not in primary lesions *in situ* [53,54]. In addition, despite chemotherapeutic drugs or radiation therapy demonstrate efficacy in 2D cultures, their activity may change significantly *in vivo*, partially affected by the TME [55,56].

In order to consider the tumor microenvironment in preclinical drug discovery, genetically engineered mouse models (GEMM) of cancer, human xenotransplant mice, or 3D organ co-cultures have been implemented. However, less than 8% of all results from animal models are reproducible in clinical cancer trials [57] and, notably, skin physiology and immunity of mice is significantly different from human skin. While melanocytes in mouse skin are mostly localized in hair follicles, determining the color of the fur, human melanocytes locate primarily at the basal layer of the epidermis, where they respond to UV-light by generating the pigment melanin and transferring it to the keratinocytes [58,59]. Thus, based on the significant differences

between mice and human skin anatomy and physiology, mouse models are limited to study melanoma progression in general and the specific role of tumor microenvironment in particular.

Hence, the research in implementing 3D models to study melanoma and its environment in three-dimensional conditions has been significantly developed. Recent advancements in 3D melanoma models are depicted in Figure 1 and chronologically listed with additional information in Table 1.

The very first 3D tumor spheroid model was generated in 1970/71 [76,77] and one of the first melanoma 3D spheroids was described 3 years later in 1973 by Folkman and Hochberg [61]. The first 3D melanoma model was based on a murine melanoma cell line (B16). In 1981, a preliminary *in vitro* full-thickness skin model was developed using rat fibroblasts seeded in a collagen lattice and littered with endothelial cells [64]. Melanoma spheroids generated directly from human tumor

material have been described in 1984 [65]. Finally, in 2000, several research groups developed different approaches for humanized 3D full-thickness melanoma models [66-68,78]. While most of them utilized human de-epidermised dermis (DED) (isolated from split thickness skin grafts or skin from skin banks) as dermal layer, seeding melanoma cells, keratinocytes and/ or fibroblasts on top [66,67,78], Meier *et al.* developed the first completely cell-based human 3D full-thickness melanoma model using fibroblasts and collagen to form a dermal basis, finally adding melanoma cells and keratinocytes on top [68]. One of the first completely cell-based human 3D full-thickness melanoma model with integrated endothelial cells and without DED as a dermal basis was developed in 2013 [70]. Furthermore, in 2013, Vörsmann *et al.* invented a human 3D full-thickness cell-based melanoma model based on integrated melanoma spheroids instead of single melanoma cells [56].

Noteworthy, to this date, fibroblast collagen I, the primary component of the ECM, derived from rat tails or bovines was

Table 1: Selected milestones in the development of 3D cutaneous melanoma models. Blue highlighted table rows are depicted in the timeline in figure 1. ECM stands for extra cellular matrix

Year	Milestone	Cell Types	ECM/ scaffold	Reference
1967	Establishment of the first human melanoma cell lines (monolayer) in cell culture	Patient-derived melanoma cell lines (LeCa 19.4, LeCa 26.5, MeGo)	-	[60]
1973	Development of the first 3D melanoma spheroid (murine cells)	Murine B-16 melanoma cells	Soft agar	[61]
1973	Establishment of the first human melanoma cell lines in suspension cell culture	Patient-derived melanoma cell lines (A-375, A-875)	-	[62]
1975	Establishment of human keratinocyte cell culture	Primary human keratinocytes co-cultured with lethal irradiated 3T3 cells	-	[63]
1981	Development of the first <i>in vitro</i> full-thickness skin model (rat cells)	Primary rat fibroblasts co-cultured with epidermal cells (rat)	Collagen lattice	[64]
1984	Development of the first human melanoma spheroids (grown directly from patient material)	Patient-derived melanoma cells	Agar coated flask	[65]
2000	Development of a human 3D full-thickness melanoma model	Primary human keratinocytes, fibroblasts, metastatic melanoma cell line (HBL)	Natural ECM of DED	[66]
2000	Development of a human 3D full-thickness melanoma model with melanoma cells from RGP, VGP or metastatic melanoma cell lines to study early melanoma metastasis	Primary human keratinocytes, melanoma cell lines from RGP (PM-WK), VGP (RPM-EP), or metastatic cell lines (MM-AN, MM-RU) No fibroblasts	Natural ECM of DED	[67]
2000	Development of the first completely cell-based human 3D full-thickness melanoma model	Primary human keratinocytes, fibroblasts, melanoma cell lines from RGP (WM35), VGP (WM793), metastatic melanoma line (WM852)	Collagen	[68]
2005	Isolation of multipotent stem-cell populations from subpopulation of spheroids of established melanoma cell lines	Established human primary melanoma cell line (WM115) and metastatic melanoma cell line (WM239A)	n/a	[69]
2013	Development of a completely cell-based human 3D full-thickness microvascularized melanoma model: Integration of a metastatic melanoma cell line induced pro-angiogenic effect on endothelial cells and lead to a more branched microvascular network	Primary human keratinocytes and fibroblasts, human umbilical vein endothelial cells (HUVEC), primary melanoma cell lines (A375, SK MEL 28), metastatic melanoma cells (Malme3, RPMI7951)	Paper anchor	[70]
2013	Development of a completely cell-based human 3D full-thickness skin melanoma spheroid model	Primary human fibroblasts, keratinocytes and tumor spheroids (451-LU cell line, MM).	Collagen I (rat tail)	[56]
2015	Development of a human 3D full-thickness melanoma invasion model with human fibroblasts stimulated to produce their own ECM constituents (collagen III, IV and VII)	Primary human fibroblasts, keratinocytes, primary human melanoma cell line (WM35) or metastatic melanoma cell line (SK-MEL-28)	Alvetex scaffold	[71]
2016	Development of a wounded and inflamed human 3D melanoma model to investigate the use of an anti-inflammatory drugs on melanoma invasion	Primary human fibroblasts, keratinocytes, metastatic melanoma cell lines (HBL, A375-SM, C8161)	Natural ECM of DED	[72]
2017	Development of a human 3D melanoma model to study melanoma invasion over time and invasion depth	Primary human fibroblasts, keratinocytes and melanoma cell line from early RGP (WM35) or metastatic melanoma cells (SK-MEL-28)	Natural ECM of DED	[73]
2018	Development of the first 3D melanoma model with blood and lymphatic capillaries	Human fibroblasts, keratinocytes, human microvascular endothelial cells (HMVEC (LEC & BEC pos.)), melanoma spheroids (A375, Malme 3 M, SK-MEL 28, RPMI 7951, WM983A, or WM983B)	Paper anchor	[40]
2019	Construction of a 3D lymph node/melanoma immune-enhanced organoid from the patient's own tumor (may allow studies for personalized immunotherapy)	Patient lymph node + melanoma tissue was shredded and digested to win the cells. All cells (unsorted) were seeded into ECM- mimicking collagen-based hydrogel	HA/collagen- based hydrogel system	[74]
2019	Development of a human 3D immune enhanced melanoma model to study young vs aged microenvironment in melanoma	Human keratinocytes, young and aged fibroblasts, melanoma cell lines (I205lu, WM3918), T-cells	Collagen	[75]

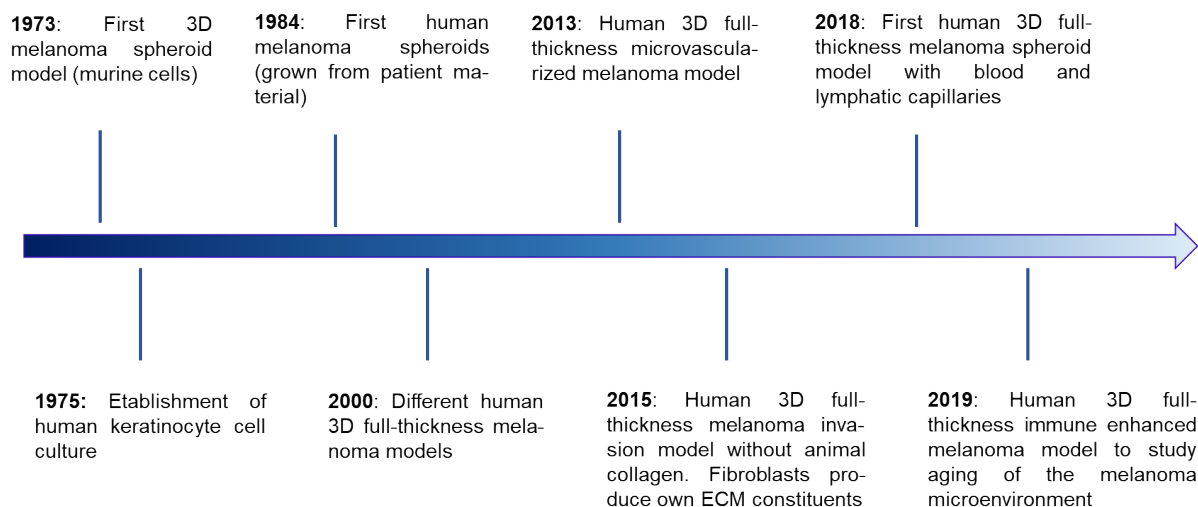


Figure 1: Timeline of selected milestones in the development of 3D cutaneous melanoma models. The details of selected milestones are listed in table 1

implemented in most cell-based models to form of the dermal layer. The first human 3D full-thickness melanoma model without animal-based collagen was described in 2013 by Gibot *et al.* [70] and in 2015 Hill *et al.* proved their model producing its own ECM constituents (collagen III, IV and VII) [71]. The first and so far only described cell-based human 3D full-thickness skin melanoma spheroid model with blood and lymphatic capillaries has been published in 2018 [40]. In this model fibroblasts were seeded onto peripheral paper anchors for sheet formation and stacked together with further separately prepared sheets consisting of human microvascular endothelial cells (HMVEC) and fibroblasts or melanoma spheroids and keratinocytes [40].

Furthermore, first approaches to develop immune enhanced 3D melanoma models have been started. For instance, one human 3D immune enhanced melanoma model with T-cells integrated in melanoma spheroids to study the young and the aged microenvironment in melanoma has been recently described [75]. Moreover, Votanopoulos *et al.* accomplished 3D lymph node/melanoma immune-enhanced organoids from the patient's own tumor, thereby reconstructing almost the exact *in vivo* situation, which may allow studies for personalized immunotherapy [74]. Importantly, while for healthy skin first human immunocompetent 3D full-thickness skin models with integrated Mutz-3, CD34⁺ or CD4⁺ T-cells have been developed [79,80], no such model has been described for melanoma yet.

Studying Invasion and Metastasis in human 3D full-thickness melanoma models

From clinical and histopathological observations in patients, melanoma progression was divided in different phases. In an initial phase, aberrant hyperproliferation of melanocytes results in formation of a benign nevi, the earliest melanocytic lesion. Further aberrant growth and dysplasia leads to the Radial Growth Phase (RGP), which is defined by intraepidermal proliferation. This phase is followed by the Vertical Growth Phase (VGP) in which the cells start to grow vertically and invade into the dermis and subcutaneous tissue, finally leading to metastasis into distant organs, particularly into the brain [81,82]. Therefore, melanoma progressive events as invasion and metastasis are complex multistep events and cannot be investigated in 2D assays.

The first human 3D full-thickness melanoma invasion models have been developed 20 years ago. These models demonstrated that

healthy melanocytes remain at the epidermal-dermal junction [66]. In addition, primary melanoma cells and melanoma cells from RGP could not invade into the dermis, but different cell lines from VGP as well as metastatic melanoma cell lines were able to cross the basal membrane and dissolve native ECM components (collagen IV and VII) [66-68,78]. Importantly, metastatic melanoma cells were only able to cross the basal membrane in the presence of keratinocytes and fibroblasts [66,83], underlining the influence of the cell-cell communication within the tumor stroma in melanoma invasion. Furthermore, invasion of melanoma cells through the basement membrane depends on the secretion of matrix metalloproteinase 9 by keratinocytes, which starts cleaving collagen type IV of the ECM after becoming activated by melanoma cells [84,85]. Notably, the invasive potential as well as the impact of fibroblasts and keratinocytes on melanoma invasion varies from cell line to cell line [73,84].

After invading the dermis and in order to detach from the primary tumor and migrate to the surrounding tissue and distant sites, melanoma cells need to change their cytoskeletal organization and communicate with the surrounding cells and ECM [86,87]. In particular, a capillary network is necessary for circulating tumor cells to adhere to microvasculature and to move across the endothelial cell layer to distant organs [88,89]. With this knowledge, research regarding melanoma TME and vascularization has been forwarded. First human 3D full-thickness melanoma models for investigations of the pro-angiogenic and pro-lymphogenic impact of melanoma cells have been described just recently. The studies of Gibot *et al.* revealed that a metastatic melanoma cell line produces high levels of vascular endothelial growth factors (VEGF) resulting in pro-angiogenic effect on endothelial cells and leading to a more complex, branched and dense microvascular network *in vitro*. These results could be confirmed by Bourland *et al.*, who developed the first human 3D full-thickness melanoma spheroid model including blood and lymphatic capillaries. The tumor spheroids secreted pro-lymphangiogenic (VEGF-C) and pro-angiogenic (VEGF-A) factors as well as CC-chemokine ligand 21 (CCL21) and angiopoietin-2 (ANG-2) in reconstructed skin. Furthermore, as the tumor surrounding cells (fibroblasts and keratinocytes) in the reconstructed skin produced a similar amount of VEGF-C, indicating that angiogenesis and lymphangiogenesis may not only be modulated by tumor cells, but also by the surrounding cells [40].

In conclusion, some cell lines are associated with RGP or advanced stages in VGP or metastasis [90], but invasion processes and progression from radial growth phase to vertical invasion and metastasis is still not fully understood and further investigations are required.

Notwithstanding, it is evident that the tumor microenvironment, cell-cell as well as cell-ECM communication are major contributors in melanoma invasion and metastasis. Thus, understanding and modeling the tumor microenvironment might be essential for therapeutic intervention in metastatic melanoma.

Conclusion

It becomes evident that monolayer cultures do not adequately recapitulate tumor biology for drug testing and that 3D models of the human skin are more appropriate models to study melanoma by considering the crucial role of its tumor microenvironment. Over the last 40 years 3D melanoma models have become increasingly sophisticated developing from mono- to co-culture and finally to vascularized and partially immunocompetent full-thickness skin models. Although various models are using reconstructed skin, most of them are based on exogenous biomaterials enriched with fibroblasts and keratinocytes. In contrast, recent 3D models based on extracellular matrix secreted by human fibroblasts, have the potential to model tumor invasion by adding human blood and lymphatic vascularization [40]. Such models may recreate a more complete microenvironment to study the effects of drug candidates and their potential to influence extravasation of tumor cells, a critical step in metastasis and a hallmark for melanoma.

However, with respect to the specific demands of innovative preclinical drug discovery, 3D model will have to manage the balancing act between robustness and miniaturization on the one hand and predictivity for the patient on the other hand. Especially, the amenability to robust medium or high throughput compound testing remains technologically challenging. Furthermore, it will be important to consider the use of primary tumor cells derived from patients instead of immortalized melanoma cell lines to characterize and identify drug candidates and select treatments for personalized medicine. In summary, despite of existing challenges, physiologically relevant *in vitro* 3D model might have the potential to partially replace animal testing according to the 3R-principle of Russell and Burch [91] by mimicking a human *in vivo*-like tumor microenvironment, potentially leading to improved clinical translation in oncology drug discovery in the future.

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