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Increased Resistance of Vasculogenic Mimicry-Forming Uveal Melanoma Cells Against Cytotoxic Agents in Three-Dimensional Cultures

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1. Introduction

Vasculogenic mimicry (VM) patterns are present in a wide variety of malignant tumors, represent the formation of perfusion pathways by tumor cells, and their presence in tumors is associated with adverse outcome (Maniotis et al., 1999; Folberg et al., 2000; Hendrix et al., 2003; Folberg and Maniotis, 2004; Döme et al., 2007). Mechanisms by which VM may contribute to adverse outcome are not well understood. Previous observations in our laboratory indicated that VM-forming tumor cells have increased resistance to herpes simplex virus-mediated oncolysis in three-dimensional (3D) uveal melanoma cultures (Valyi-Nagy et al., 2010). To determine whether VM-forming tumor cell subpopulations also have increased resistance against cytotoxic drugs, traditional two-dimensional (2D) and extracellular matrix (ECM, Matrigel)-containing 3D cultures of C918 uveal melanoma cells were established. In 2D cultures, C918 cells grew in monolayers. In 3D cultures, C918 cells formed a number of morphologically distinct tumor cell subpopulations that included cells that grew in monolayers on the Matrigel surface, cells that formed VM patterns, and cells that formed monolayers on the bottom of the culture dish. Following exposure to cisplatin or cadmium chloride, VM-forming tumor cells demonstrated prolonged survival relative to other tumor cell subpopulations in 3D cultures and cells grown in 2D. As presented in detail below, these findings suggest that increased drug resistance is a mechanism by which VM-forming tumor cells contribute to adverse outcome.

1.1 Vasculogenic mimicry

VM patterns represent the formation of perfusion pathways by tumor cells (Maniotis et al., 1999). VM is composed of patterned networks of laminin-rich basement membranes lined by tumor cells. VM patterns are present in a wide variety of malignant tumors and the presence of VM in tumors is associated with adverse outcome (Folberg et al., 1992 and 1993; Folberg and Maniotis, 2004; Gosh et al., 2005; Folberg et al., 2000 and 2007; Hendrix et al., 2003; Döme et al., 2007). While VM formation is clearly a marker of highly invasive tumor phenotype, mechanisms by which these structures may contribute to adverse outcome are not well understood. It has been proposed that VM formation may facilitate tumor

perfusion and the physical connection between VM and blood vessels may also facilitate hematogeneous dissemination of tumor cells (Folberg and Maniotis, 2004). Interestingly, uveal melanoma cells within VM patterns assume a spindle A morphology and the expression of the Ki67 proliferation marker is significantly reduced in these cells (Folberg et al., 2006). These findings raise the possibility that VM-forming tumor cells have increased resistance against radiation and chemotherapeutic agents that target highly proliferative tumor cell populations.

Recent studies also indicate that malignant melanoma initiating cells (MMIC) are specifically associated with VM and it has been proposed that one mechanism by which MMIC promote tumor growth is by the induction of VM formation by MMIC (Frank et al., 2011). Previous work in our laboratory indicated that VM-forming tumor cells in 3D tumor cell cultures have increased resistance against at least one form of therapy: oncolytic virotherapy (Valyi-Nagy et al., 2010). To test the hypothesis that VM-forming tumor cells also have increased resistance against cytotoxic agents, in the current study we utilized 3D cultures of uveal melanoma cells as an experimental platform.

1.2 Three-dimensional tumor cultures

The introduction of 3D tumor cultures has revolutionized anticancer drug research as these cultures allow for the study of drug resistance mechanisms that can not be explored in traditional two dimensional (2D) monolayer cultures. The behavior of cells *in vivo* is controlled by their interactions with neighboring cells and with the ECM (Abbott, 2003; Friedrich, 2003; Nelson and Bissel, 2004; Smalley et al., 2006; Schmeichel and Bissel, 2005; Sandal et al., 2007; Wang et al., 1988; Weaver et al., 1997). Cancer cells grown in 3D cultures in a polymeric ECM closely mimic the biology of tumor development in vivo and numerous studies indicate that 3D cultures are superior to traditional 2D monolayer cultures for studies of key cellular behaviors like differentiation, proliferation, invasion and apoptosis (Xu and Burg, 2007). Cancer cells grown in 3D culture are more resistant to chemotherapeutic agents and radiation than cells in 2D culture and 3D tumor cell cultures are useful for preclinical evaluation of the cytotoxic effect of anticancer agents (Smalley et al., 2006). It is well established that multiple cell types within individual tumors have differential sensitivities to drugs and radiation both *in vivo* and in 3D cultures (Schmidmaier and Baumann, 2008; Jacks and Weinberg, 2002; Vescio et al., 1987).

Uveal melanoma cells form several morphologically distinct cell populations under 3D culture conditions (Maniotis et al., 1999, 2005; Valyi-Nagy et al., 2010). Importantly for our current study, the several morphologically distinct cell populations formed by highly invasive uveal melanoma cells under 3D culture conditions include cells that form VM (Maniotis et al., 1999; Folberg and Maniotis, 2004). To determine whether VM-forming tumor cell subpopulations have increased resistance against cytotoxic drugs, traditional two-dimensional (2D) and extracellular matrix (Matrigel)-containing 3D cultures of C918 uveal melanoma cells were established and were then exposed to cytotoxic agents.

2. Experimental approach

2.1 Cells

C918 uveal melanoma cells of high invasive potential were maintained in Eagle's Minimal Essential Medium (EMEM, BioWhittaker Inc., Walkersville, MD) supplemented with heat

inactivated 15% fetal bovine serum (Fisher, Ontario, Canada) without the addition of exogenous extracellular matrix molecules or growth factors. This cell line has been described in detail previously (Folberg et al., 2008).

2.2 Establishment of 2D and 3D melanoma cultures

For the establishment of 2D and 3D cultures used in experiments, C918 uveal melanoma cells were grown on 24-well plates in EMEM medium either in the presence (3D cultures) or in the absence (2D cultures) of extracellular matrix rich in laminin (Matrigel, BD Biosciences, Bedford, MA) as described previously (Valyi-Nagy et al., 2007 and 2010). For 3D cultures, Matrigel was poured onto the bottom of the tissue culture wells to a depth of approximately 0.2 mm and was allowed to polymerize for 1 hour at 37^{o}C. Following polymerization, C918 melanoma cells were placed on the Matrigel surface and the cultures were incubated in repeatedly refreshed culture medium for up to 4 weeks. Cultures were observed daily under an inverted microscope (Leica, Bannockburn, IL).

2.3 Exposure of 2D and 3D melanoma cultures to cytotoxic agents

Cells were first exposed to various concentrations of cytotoxic agents four to five days after the initiation of cultures, when 2D cultures formed monolayers and cells in 3D cultures formed morphologically distinct tumor cell subpopulations including VM patterns. Tested cytotoxic agents included cisplatin [cis-diammineplatinum(II)dichloride, Sigma] and cadmium chloride [$CdCl_2$ hemi (pentahydrate), Sigma]. Culture media containing either cisplatin or cadmium chloride were changed daily. Cultures were observed daily under an inverted fluorescence microscope (Leica, Bannockburn, IL) for evidence of drug toxicity. The day when at least 99% of a given morphologically distinct tumor cell subpopulation was destroyed was noted. Cell death was confirmed by the uptake of the charged cationic dye Trypan blue (Mediatech Inc., Manassas, VA) by more than 99% of cells following incubation of cultures with Trypan blue (0.2%) for 10 minutes at 37 °C.

3. Results

3.1 Uveal melanoma cells form morphologically distinct cell populations under 3D conditions

As expected based on previous observations (Valyi-Nagy et al, 2010), C918 cells grew in monolayers in 2D cultures (Fig.1) and formed several morphologically distinct cell populations under 3D culture conditions (Fig.2). In 3D cultures, morphologically distinct tumor cell subpopulations included cells that grew in monolayers on the Matrigel surface, cells that formed VM patterns, and cells that formed monolayers on the bottom of the culture dish (Fig.2). The growth of 3D cultures demonstrated the following course: when C918 cells were placed on the Matrigel surface, the cells started to grow on the Matrigel surface in a single layer and focally formed circular vasculogenic mimicry patterns that surrounded round matrix surfaces free of tumor cells. Next, tumor cells started to grow into the Matrigel at the line defined by the vasculogenic mimicry patterns and migrated to the bottom of the culture plate where they formed monolayers. The formation of these bottom monolayers was restricted to areas that were in the center of VM patterns (Fig. 2).

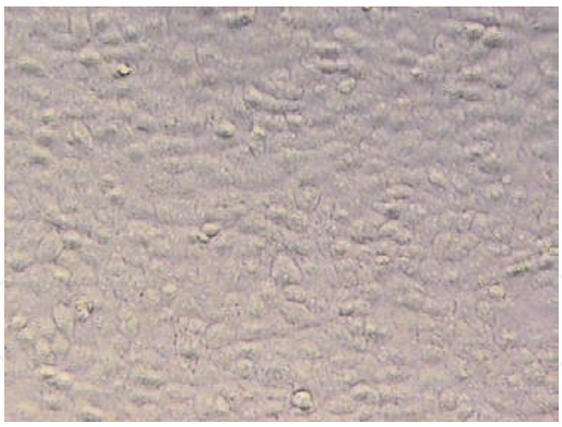

Fig. 1. Morphology of 2D C918 uveal melanoma cultures. In traditional 2D cultures, C918 uveal melanoma cells formed a monolayer of cells on the bottom surface of the tissue culture wells (200x magnification).

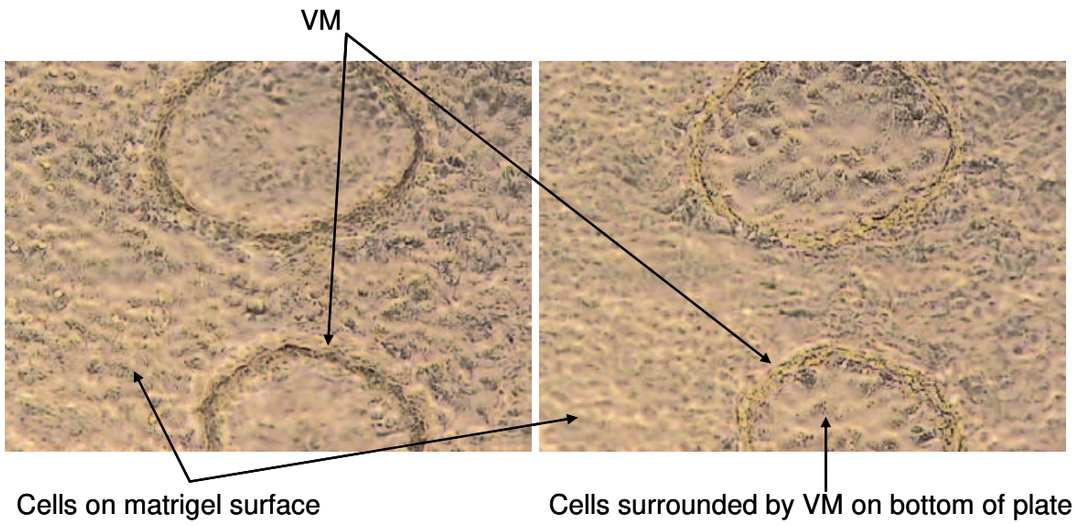

Fig. 2. Morphology of 3D C918 uveal melanoma cultures. Pictures of a 3D C918 uveal melanoma culture with focus either on cells growing on the Matrigel surface (left panel) or on cells growing on the bottom of the tissue culture well (right panel). Vasculogenic mimicry (VM) patterns are also marked by arrows (200x magnification).

3.2 VM-forming melanoma cells have increased resistance against cisplatin

Starting 4 to 5 days after the initiation of cultures, when 2D cultures formed monolayers and cells in 3D cultures formed morphologically distinct tumor cell subpopulations including VM patterns, cultures were exposed to various concentrations of cisplatin (Del Bello et al., 2003; Feldman et al., 2004; Bowden et al., 2010) and observed daily for toxicity. Cisplatin concentrations tested in the experiments ranged from 30 to 300 μM and fresh culture media containing cisplatin were added to the cultures daily. Control cultures not exposed to cisplatin remained viable and demonstrated growth. However, cultures exposed to cisplatin demonstrated dose dependant toxicity (Table 1). Interestingly, we found that VM-forming tumor cells demonstrated prolonged survival following cisplatin treatment relative to other

tumor cell subpopulations in 3D cultures and relative to cells grown in 2D (Table 1, Figs 3 to 8). For instance, it took 6 days until 300 μM cisplatin destroyed more than 99% of cells forming VM in 3D cultures while other cell subpopulations in 3D cultures and cells grown in 2D cultures were destroyed by 4 and 3 days, respectively (Table 1). Even more pronounced differences were detected at 100 μM cisplatin concentrations as destruction of VM-forming tumor cells never reached 99% in 3D cultures during a 7-day observation period while other cell subpopulations in 3D cultures and cells grown in 2D cultures were destroyed by 4 and 3 days, respectively (Table 1). These findings indicate that VM-forming tumor cells have increased resistance against the cytotoxic effects of cisplatin in 3D uveal melanoma cultures.

	2D	**3D**		
	Cells on surface of plate	Cells on surface of Matrigel	VM	Cells on surface of plate
No CPL Control	NA (>7 days)	NA (>7 days)	NA (>7 days)	NA (>7 days)
CPL 30 μM	NA (>7 days)	NA (>7 days)	NA (>7 days)	NA (>7 days)
CPL 100 μM	3 days	4 days	NA (>7 days)	4 days
CPL 300 μM	3 days	4 days	6 days	4 days

Table 1. Time (days) elapsed from the initiation of cisplatin treatment to destruction of more than 99% of C918 uveal melanoma cells in 2D cultures and destruction of more than 99% of various C918 cell subpopulations in 3D cultures during a 7-day observation period. Abbreviations: 2D = two-dimensional cultures; 3D = three-dimensional cultures; VM = cells forming vasculogenic mimicry patterns; CPL = cisplatin; NA (>7 days) = not applicable, cell death did not reach 99% during the 7-day observation period.

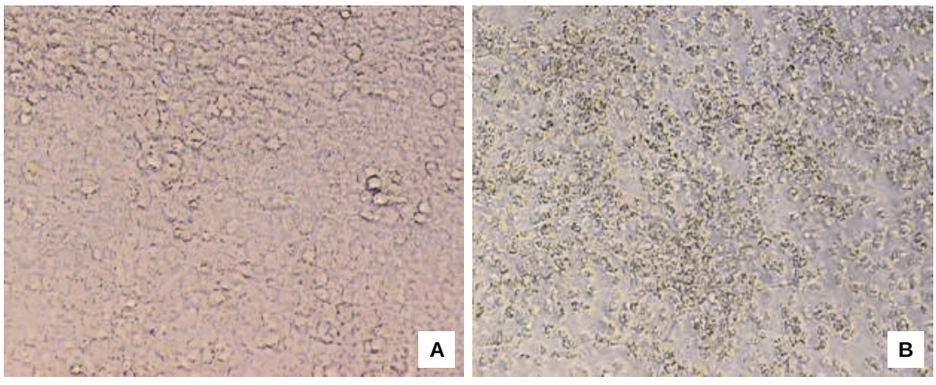

Fig. 3. Destruction of 2D C918 uveal melanoma cultures following exposure to cisplatin for four days. Panel A shows cultures grown in regular culture medium. Panel B shows destruction of cells that were cultured in medium containing 100 μM cisplatin. Magnification: 100x.

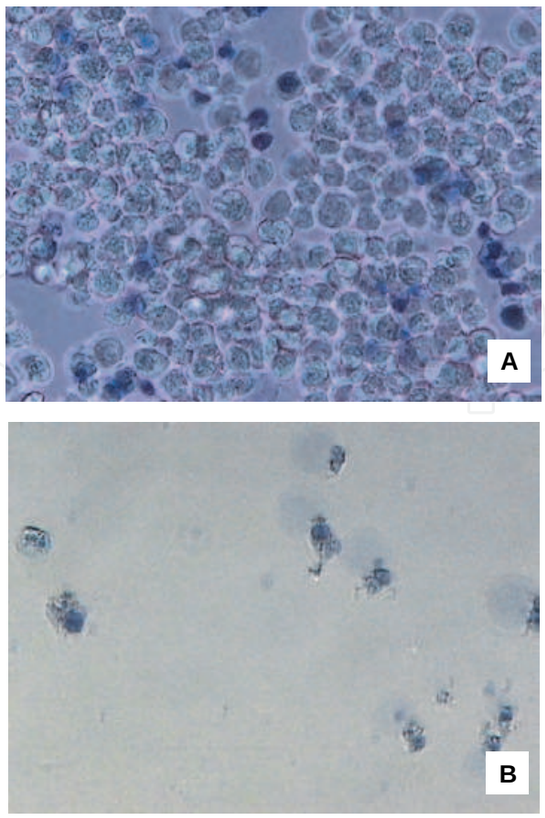

Fig. 4. Trypan blue staining in 2D C918 uveal melanoma cultures following 100 μM cisplatin treatment for seven days. While the majority of cells are viable (negative for trypan blue) in untreated control cultures (panel A), no viable (trypan blue-negative) C918 uveal melanoma cells are detected after 100 μM cisplatin treatment (panel B) (200x magnification).

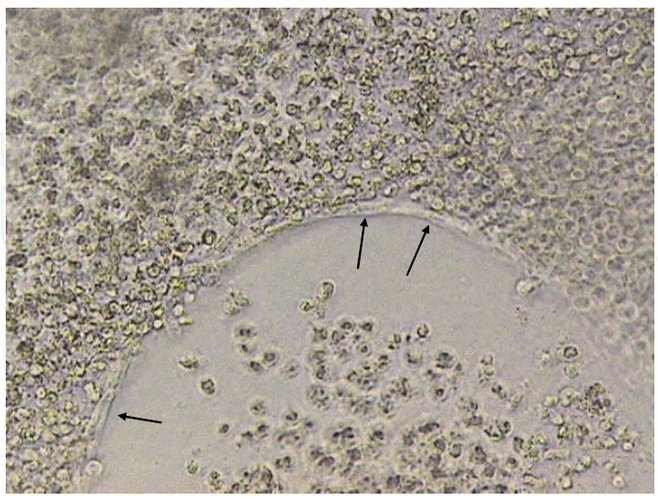

Fig. 5. Effect of 100 μM cisplatin on 3D C918 uveal melanoma cultures. All cells but those forming VM patterns demonstrate extensive destruction by 100 μM cisplatin in 3D cultures of C918 uveal melanoma cells three days after initiation of cisplatin treatment. Arrows point to VM (100x magnification).

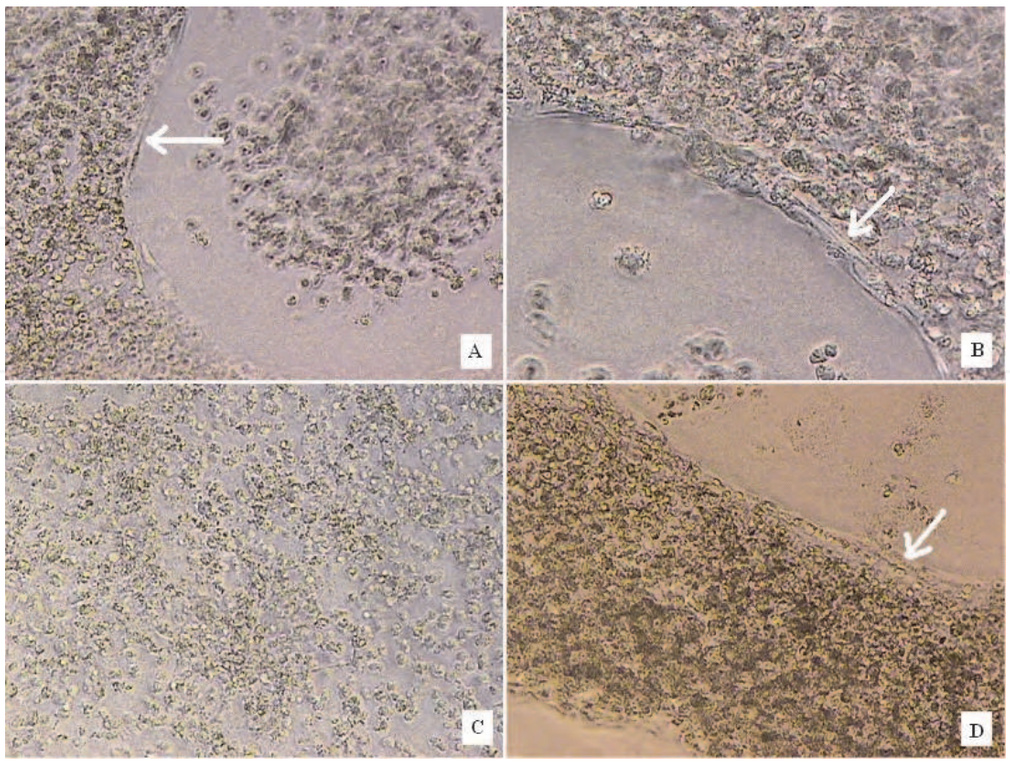

Fig. 6. Effect of 100 μM cisplatin on the morphology of 2D and 3D C918 uveal melanoma cultures. All cells but those forming VM patterns (arrows) are destroyed by 100 μM cisplatin in 3D cultures exposed for four days to cisplatin (panels A, B, and D). Destruction of 2D cultures exposed for four days to cisplatin (panel C). Magnification: 100x.

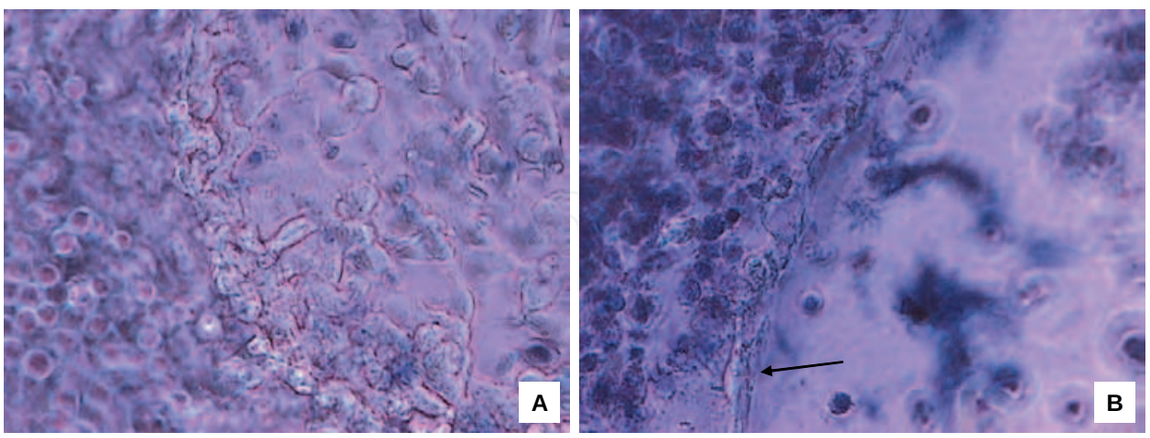

Fig. 7. Trypan blue staining in 3D C918 uveal melanoma cultures exposed for four days to regular medium (panel A) or to medium containing 100 μM cisplatin (panel B). Note that the majority of cells are viable (negative for trypan blue) in the control cultures in panel A. In contrast, only some of the cells associated with VM formation are viable (negative for trypan blue) in 3D cultures four days after 100 μM cisplatin treatment. Arrow in panel B points to VM (200x magnification).

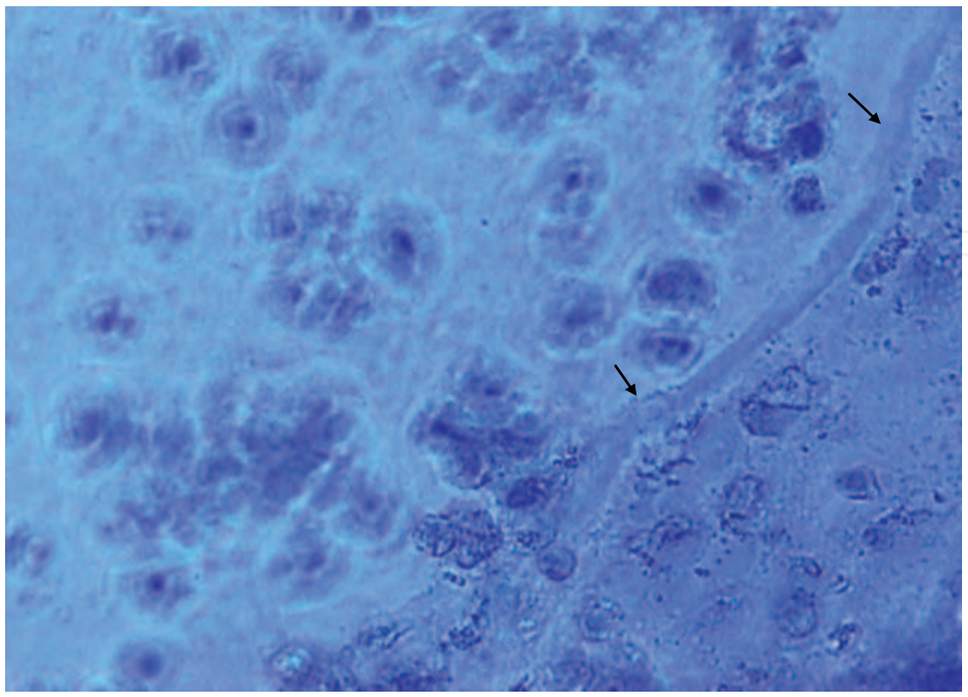

Fig. 8. Trypan blue staining in a 3D C918 uveal melanoma culture following 100 μM cisplatin treatment for seven days. Only some of the cells associated with VM formation (arrows) are viable (negative for trypan blue). Magnification: 200x.

3.3 VM-forming melanoma cells have increased resistance against cadmium

Starting 4 to 5 days after the initiation of cultures, when 2D cultures formed monolayers and cells in 3D cultures formed morphologically distinct tumor cell subpopulations including VM patterns, cultures were exposed to various concentrations of cadmium chloride (Yokouchi et al., 2007) and observed daily for toxicity. Cadmium chloride concentrations tested in the experiments ranged from 20 to 1000 μM. Fresh culture media containing cadmium chloride were added to the cultures daily for up to 4 weeks. Control cultures not exposed to cadmium chloride remained viable and demonstrated growth for several weeks. However, cultures exposed to cadmium chloride in the culture medium demonstrated dose dependant toxicity (Table 2). Similarly to the observations made with cisplatin, VM-forming tumor cells demonstrated prolonged survival following cadmium treatment relative to other tumor cell subpopulations in 3D cultures and cells grown in 2D (Table 2, Figs 9 through 14). Specifically, at 200 μM cadmium chloride concentration, destruction of VM-forming tumor cells never reached 99% in 3D cultures during a 16-day observation period while other cell subpopulations in 3D cultures and cells grown in 2D cultures were completely destroyed by 4 and 3 days, respectively (Table 2). Interestingly, as presented in the next section in detail, if cadmium chloride treatment was stopped sixteen days after initiation of drug treatment, residual still viable cells in VM patterns served as foci of new tumor growth (Fig. 16 through 18). These findings indicate that VM-forming tumor cells have increased resistance against the cytotoxic effects of cadmium chloride in 3D uveal melanoma cultures.

	2D	3D		
	Cells on surface of plate	Cells on surface of Matrigel	VM	Cells on surface of plate
No Cd Control	NA (>16 days)	NA (>16 days)	NA (>16 days)	NA (>16 days)
Cd 20 µM	NA (>16 days)	NA (>16 days)	NA (>16 days)	NA (>16 days)
Cd 200 µM	4 days	4 days	NA (>16 days)	4 days
Cd 400 µM	2 days	2 days	2 days	2 days
Cd 1000 µM	2 days	2 days	2 days	2 days

Table 2. Time (days) elapsed from the initiation of cadmium chloride ($CdCl_2$) treatment to destruction of more than 99% of C918 uveal melanoma cells in 2D cultures and destruction of more than 99% of various C918 cell subpopulations in 3D cultures during a 16-day observation period. Abbreviations: 2D = two-dimensional cultures; 3D = three-dimensional cultures; VM = cells forming vasculogenic mimicry patterns; Cd = cadmium chloride ($CdCl_2$); NA (>16 days) = not applicable, cell death did not reach 99% during the 16-day observation period.

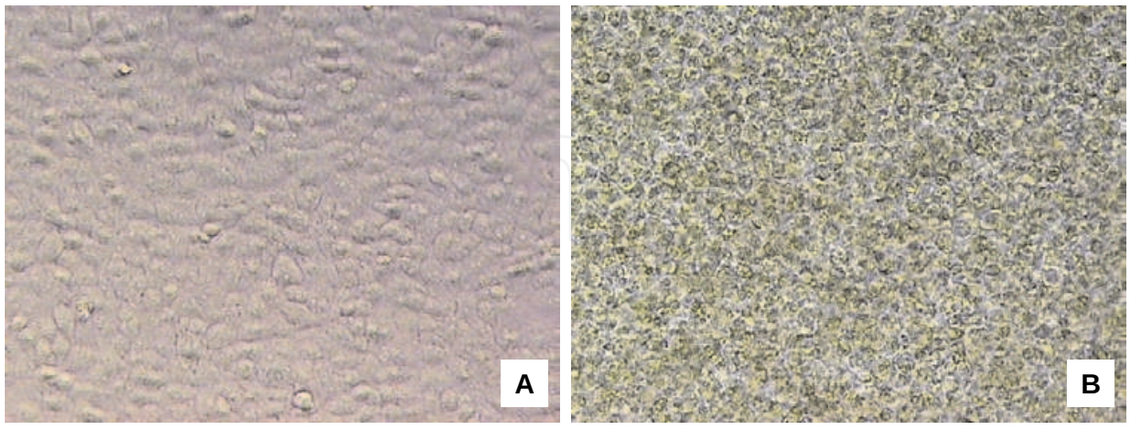

Fig. 9. Effect of 200 µM cadmium chloride on 2D C918 uveal melanoma cultures. Panel A shows cultures grown in regular culture medium. Panel B shows destruction of cells that were cultured in medium containing 200 µM cadmium chloride for four days. Magnification: 100x.

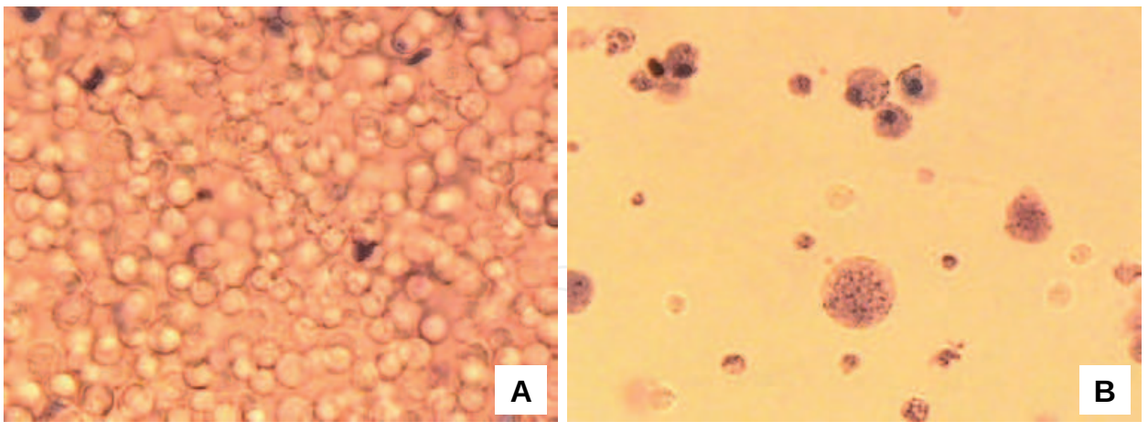

Fig. 10. Trypan blue staining in 2D C918 uveal melanoma cultures following 200 μM cadmium treatment for four days. While the majority of cells are viable (negative for trypan blue) in untreated control cultures (panel A), no viable (trypan blue-negative) C918 uveal melanoma cells are detected after 200 μM cadmium treatment (panel B) (200x magnification).

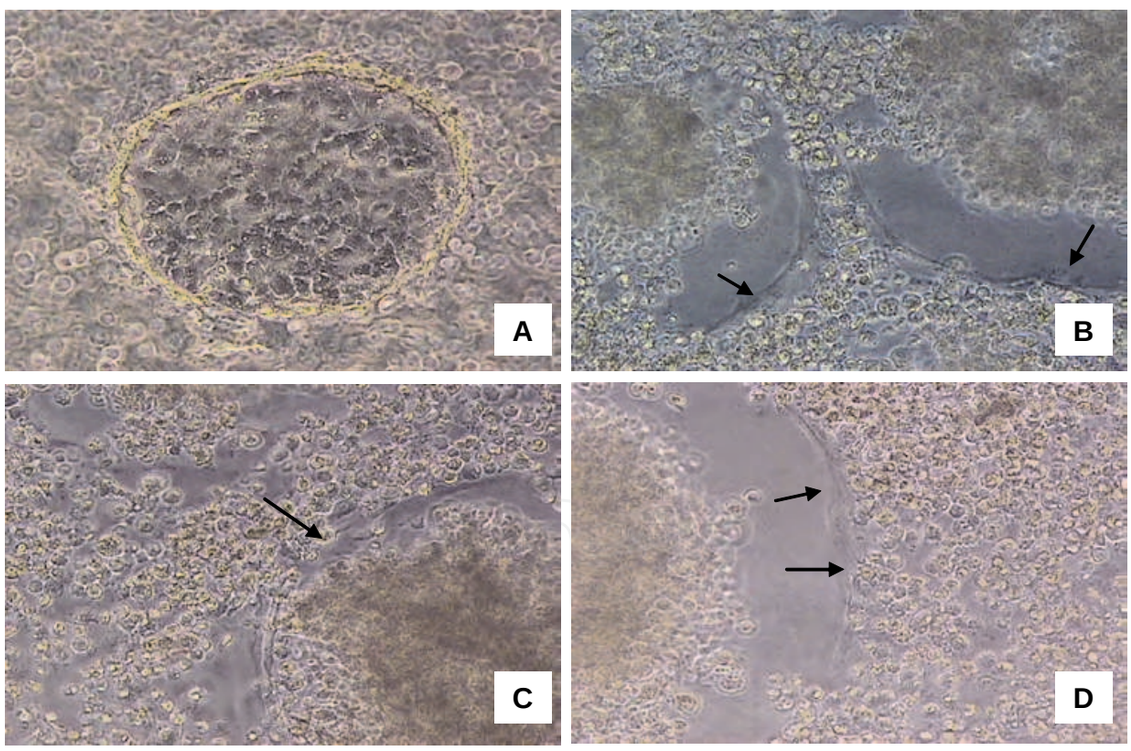

Fig. 11. Effect of 200 μM cadmium chloride on 3D C918 uveal melanoma cultures. Panel A shows a 3D culture grown in regular medium for nine days and is notable for an abundance of viable-appearing cells. Panels B through D show 3D cultures grown in medium containing 200 μM cadmium chloride for nine days. Note that all but some cells forming VM patterns are destroyed by the cadmium chloride treatment. Arrows point to VM in panels B, C, and D. Magnification: 200x.

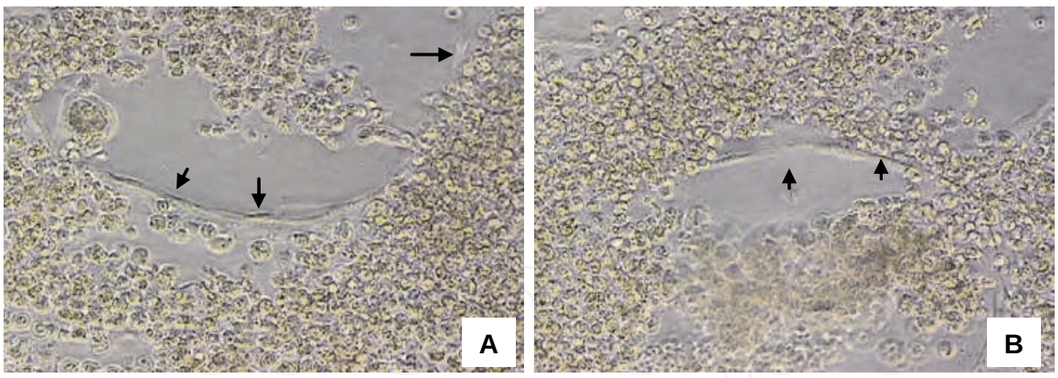

Fig. 12. Effect of exposure of 3D C918 uveal melanoma cultures to 200 µM cadmium chloride for nine days (A) or ten days (B). Note that all but some cells forming VM patterns (arrows) are destroyed by the cadmium chloride treatment. Magnification: 200x.

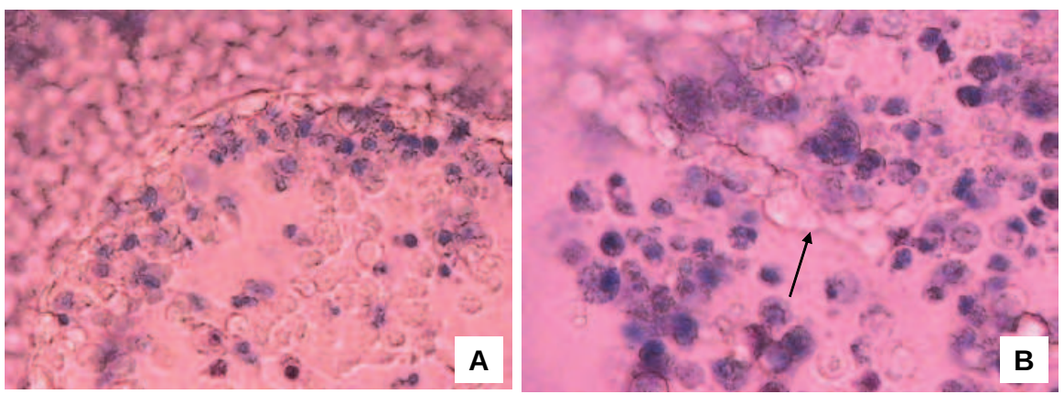

Fig. 13. Trypan blue staining in 3D C918 uveal melanoma cultures exposed for four days to regular medium (A) or to medium containing 200 µM cadmium chloride (B). The majority of cells are viable in the control cultures (A). Only some cells associated with VM formation are viable (negative for trypan blue) after cadmium treatment (arrow) (200x magnification).

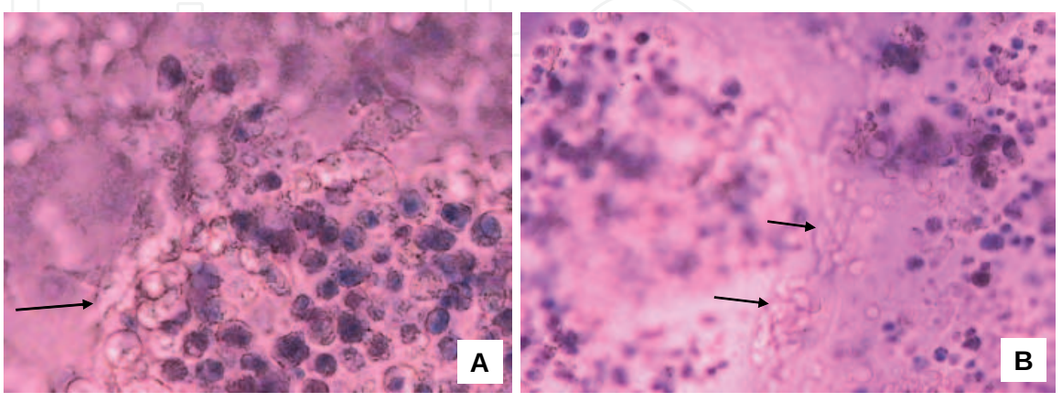

Fig. 14. Trypan blue staining in 3D C918 uveal melanoma cultures exposed for four days to 200 µM cadmium chloride. Only some tumor cells associated with VM formation (arrows) are viable (negative for trypan blue). 200x magnification.

3.4 VM-forming melanoma cells may serve as foci of renewed growth once drug treatment is stopped

Findings presented above indicate that VM-forming tumor cells have increased resistance against the cytotoxic effects of cadmium chloride in 3D uveal melanoma cultures. Interestingly, if cadmium chloride treatment was stopped sixteen days after initiation of drug treatment, residual still viable cells associated with VM patterns appeared to serve as foci of new tumor growth in the 3D cultures (Fig. 15 through 18).

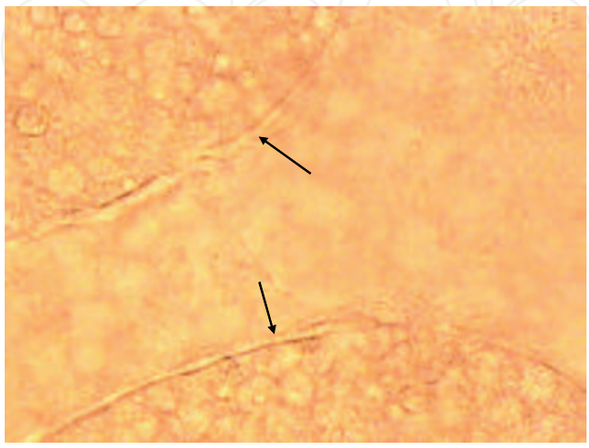

Fig. 15. Morphology of a 3D C918 melanoma culture one day after cadmium chloride treatment was stopped. Three-dimensional cultures of C918 uveal melanoma cells were exposed for sixteen days to medium containing 200 µM cadmium chloride resulting in the destruction of all cells but some cells forming VM (arrows). There is no evidence of cell growth from the VM yet. Magnification: 200x.

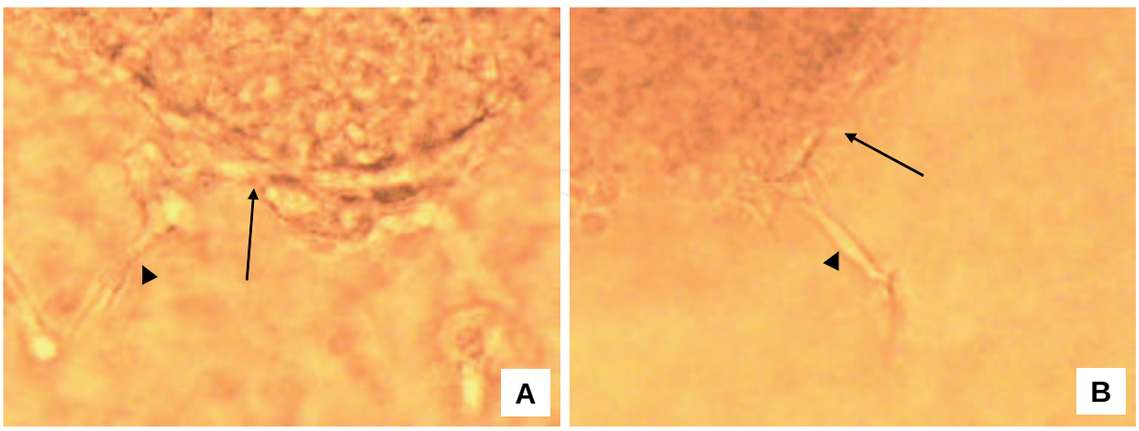

Fig. 16. Cells growing out from VM patterns four days following withdrawal of cadmium chloride treatment. Three-dimensional cultures of C918 uveal melanoma cells were exposed for sixteen days to medium containing 200 µM cadmium chloride resulting in the destruction of all cells but some forming VM (arrows). Note focus of resumed melanoma cell growth (arrowheads) from VM. Magnification: 200x.

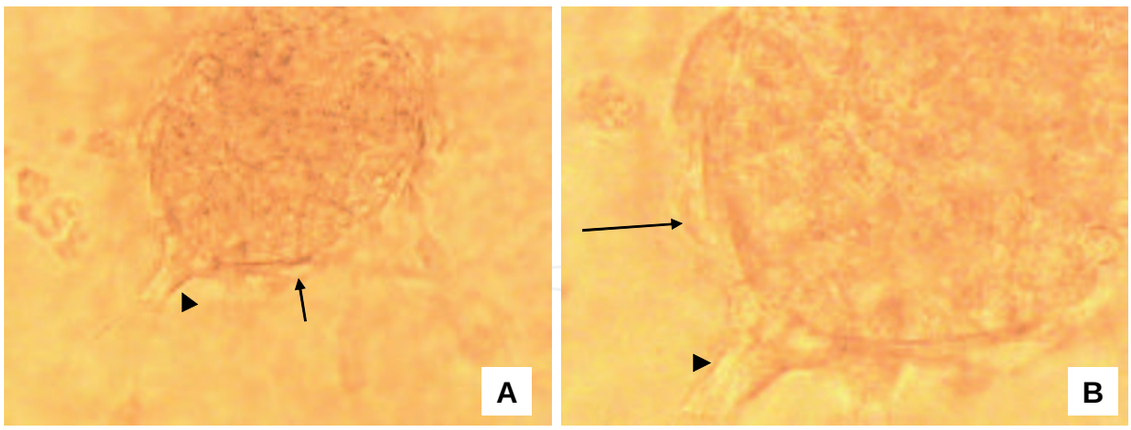

Fig. 17. Cells growing out from VM patterns four days following withdrawal of cadmium chloride treatment. Three-dimensional cultures of C918 uveal melanoma cells were exposed for sixteen days to medium containing 200 µM cadmium chloride resulting in the destruction of all cells but some forming VM (arrows). Note focus of resumed melanoma cell growth (arrowheads) from VM. Magnification: 100x (A), 200x (B).

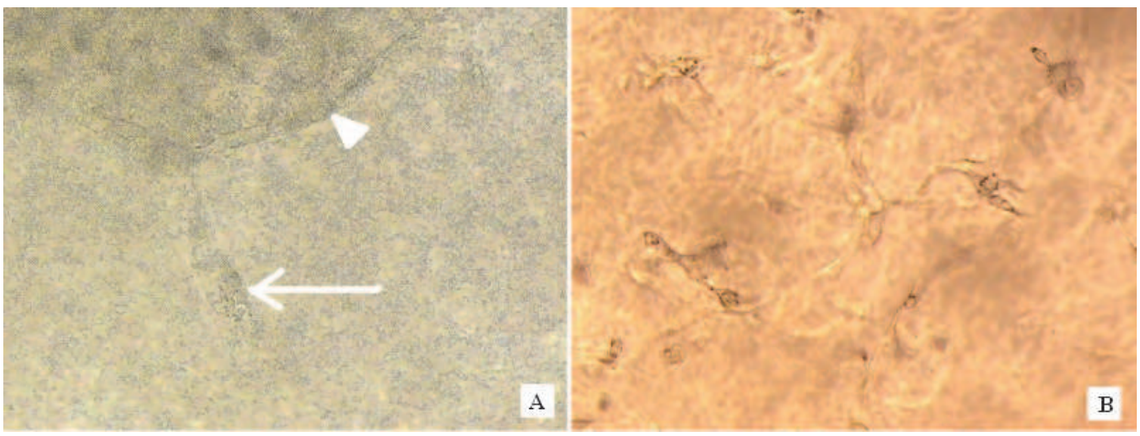

Fig. 18. Cells growing out from VM patterns following withdrawal of cadmium chloride treatment. Three-dimensional cultures of C918 uveal melanoma cells were exposed for sixteen days to medium containing 200 µM cadmium chloride resulting in the destruction of all cells but some of those associated with VM. Panel A shows cultures two days after drug withdrawal. Focus of resumed melanoma cell growth (arrow) is associated with VM (arrowhead). Panel B shows cultures nine days after drug withdrawal (200 x magnification).

4. Conclusions

While VM formation is clearly a marker of highly invasive tumor phenotype *in vivo*, mechanisms by which VM may contribute to adverse outcome are not well understood (Folberg et al., 1993; Maniotis et al., 1999; Folberg et al., 2000; Hendrix et al., 2003; Folberg and Maniotis, 2004; Lin et al., 2005; Döme et al, 2007). We have shown here that in 2D cultures, C918 uveal melanoma cells grow in monolayers and in 3D cultures, C918 cells form a number of morphologically distinct tumor cell subpopulations that include cells that form

VM patterns. Importantly, we found that following exposure to cytotoxic agents, VM-forming tumor cells demonstrate prolonged survival relative to other tumor cell subpopulations in 3D cultures and cells grown in 2D. Additional observations suggested that drug resistant VM-forming tumor cells may serve as foci of new tumor growth once cytotoxic drug levels drop. While confirmation of these observations in *in vivo* studies and other tumor types will be essential, our findings reported here suggest that increased drug resistance is a mechanism by which VM-forming tumor cells contribute to adverse outcome. As VM patterns have been described in a wide variety of malignant neoplasms, observations of this current report are of great potential significance.

5. Acknowledgments

This work was supported by fellowship grants to Andras Voros and Eva Gagyi by the Rosztoczy Foundation.

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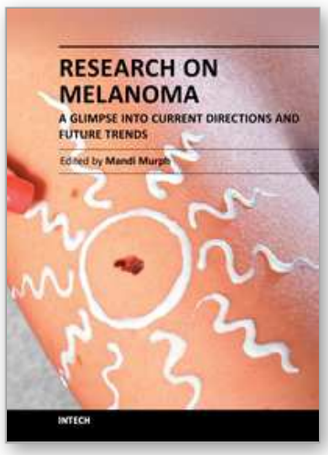

Research on Melanoma - A Glimpse into Current Directions and Future Trends

Edited by Prof. Mandi Murph

ISBN 978-953-307-293-7
Hard cover, 414 pages
Publisher InTech
Published online 12, September, 2011
Published in print edition September, 2011

The book Research on Melanoma: A Glimpse into Current Directions and Future Trends, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

How to reference

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Klara Valyi-Nagy, Andras Voros, Eva Gagyi and Tibor Valyi-Nagy (2011). Increased Resistance of Vasculogenic Mimicry-Forming Uveal Melanoma Cells against Cytotoxic Agents in Three-Dimensional Cultures, Research on Melanoma - A Glimpse into Current Directions and Future Trends, Prof. Mandi Murph (Ed.), ISBN: 978-953-307-293-7, InTech, Available from: http://www.intechopen.com/books/research-on-melanoma-a-glimpse-into-current-directions-and-future-trends/increased-resistance-of-vasculogenic-mimicry-forming-uveal-melanoma-cells-against-cytotoxic-agents-i

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