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Adaptation to ER Stress as a Mechanism of Resistance of Melanoma to Treatment

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1. Introduction

A number of cellular stress conditions, such as nutrient deprivation, hypoxia, alterations in glycosylation status, and disturbances of calcium flux, lead to accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen and cause so-called ER stress. The ER responds to the stress conditions by activation of a range of stress-response signalling pathways to alter transcriptional and translational programs, which couples the ER protein folding load with the ER protein folding capacity and is termed the unfolded protein response (UPR).

The UPR of mammalian cells is initiated by three ER transmembrane proteins - activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and double-stranded RNA-activated protein kinase-like ER kinase (PERK) that act as proximal sensors of ER stress. Under unstressed conditions, the luminal domains of these sensors are occupied by the ER chaperone glucose-regulated protein 78 (GRP78). Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors by inducing phosphorylation and homodimerization of IRE1 and PERK, and relocalization of ATF6 to the Golgi where it is cleaved by Site 1 and Site 2 proteases (S1P and S2P) leading to its activation as a transcriptional factor. The UPR is fundamentally a cyto-protective response, but excessive or prolonged UPR can result in cell death, predominantly by induction of apoptosis.

Nevertheless, most melanoma cell lines are insensitive, relative to cultured melanocytes and fibroblasts, to apoptosis induced by pharmacological ER stress inducers, suggesting that melanoma cells may have developed adaptive mechanisms to counteract the apoptosis-inducing outputs of the UPR, thus surviving ER stress conditions. This is supported by increased expression of GRP78, a commonly used indicator of activation of the UPR, in melanoma cells *in vivo*, which is associated with progression of the disease. It is conceivable that, like many other solid cancers, melanoma cells in a developing tumour without sufficient blood supply may undergo hypoxia, nutrient starvation and acidosis. In addition, accumulation of mutant proteins and increased glycolytic activity in cancer cells may also contribute to ER stress.

How the UPR switches between the pro-survival and pro-apoptotic signalling pathways remains a dilemma. It is conceivable that adaptive processes converge on mechanisms that

inhibit apoptosis. In this chapter, we will discuss multiple mechanisms that are involved in resistance of melanoma cells to apoptosis induced by ER stress. These include persistent activation of the IRE1 branch of the UPR that is responsible for up-regulation of the anti-apoptotic Bcl-2 family protein Mcl-1, and dysregulation of CHOP, a transcription factor down-stream of the PERK branch of the UPR that otherwise triggers apoptosis by up-regulation of the BH3-only protein Bim. We will also present evidence showing important roles of activation of other survival pathways such as the MEK/ERK and PI3K/Akt pathways in adaptation of melanoma cells to ER stress.

Two fundamental questions that are directly relevant to clinical management of melanoma are how adaptation to ER stress impacts on responses of melanoma cells to treatment and whether induction of ER stress by therapeutic agents is beneficial or detrimental. Melanoma cells under pharmacological ER stress acquire resistance to apoptosis induced by microtubule-targeting drugs docetaxel and vincristine, suggesting that activation of the UPR protects melanoma cells against these agents. On the other hand, induction of apoptosis by a number of chemotherapeutic drugs, such as the DNA damaging drugs cisplatin and adriamycin, the proteasome inhibitor bortezomib, and the synthetic retinoid derivative fenretinide, is known to be associated with induction of ER stress. However, there is evidence showing that inhibition of the UPR effector GRP78 sensitizes melanoma cells to cisplatin and adriamycin. We will discuss possible explanations for these seemingly paradoxical observations in this chapter, and will propose that induction of ER stress by therapeutic agents is a double-edged sword. We will also present data demonstrating that targeting adaptive mechanisms to ER stress may be useful in the treatment of melanoma, especially in combination with agents that induce ER stress.

2. ER stress and the UPR

The ER is an organelle of a lacey network of cisternae that has essential roles in many cellular processes required for cell survival, growth and other functions. These include intracellular calcium homeostasis, protein folding and glycosylation, and lipid biosynthesis (Gaut & Hendershot, 1993). Moreover, because the ER releases calcium through ion channels in response to second messengers such as inositol triphosphate (IP3) and protein kinases, it is also regarded as a signalling organelle (Schröder & Kaufman, 2005).

ER stress is a condition under which intracellular or extracellular disturbances, such as nutrient deprivation, hypoxia, alterations in glycosylation status, disturbances of calcium flux, cause accumulation and aggregation of unfolded and/or misfolded proteins in the ER lumen (Ma & Hendershot, 2004; Ron & Walter, 2007; Schröder & Kaufman, 2005). Cells undergoing ER stress respond to protect themselves by activating the UPR, which alters transcriptional, translational, and post-translational programs, resulting in up-regulation of ER chaperones, general translational attenuation, and enhanced ER-associated degradation (ERAD) of misfolded and unfolded proteins. The signalling pathways of the UPR are initiated by three ER transmembrane proteins, ATF6, IRE1, and PERK (Ma & Hendershot, 2004; Ron & Walter, 2007; Schröder & Kaufman, 2005). Under unstressed conditions, the luminal domains of these sensors are occupied by ER chaperone proteins, in particular, GRP78. Upon ER stress, increased binding of GRP78 with misfolded and unfolded proteins causes removal of GRP78 from ATF6, IRE1, and PERK, thus resulting in release of these proximal ER stress sensors and activation the UPR (Fig. 1). GRP78 has therefore been termed the "master regulator" of the UPR (Hendershot, 2004; Lee, 2005).

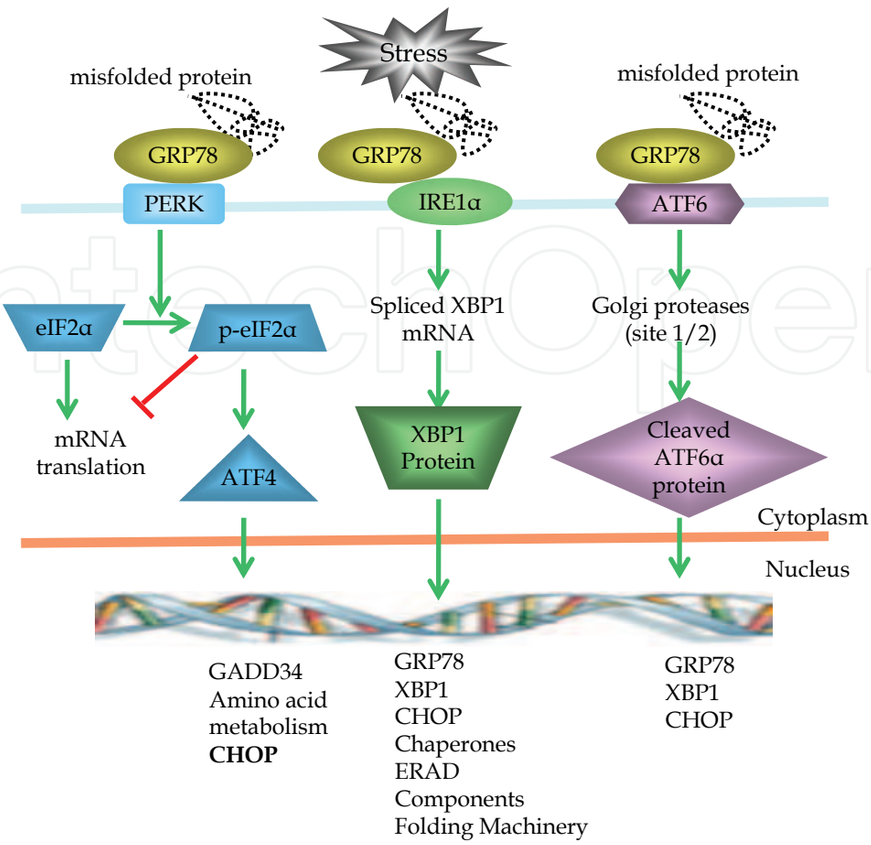

Fig. 1. A schematic illustration of signalling pathways of the UPR

2.1 IRE1α

IRE1α is an unusual protein in that it acts as both a serine/threonine protein kinase and an endoribonuclease (Ma & Hendershot, 2004; Ron & Walter, 2007; Schröder & Kaufman, 2005). The latter activity processes an intron leading to the catalytic removal of a 26-base intron from the mRNA of the gene, *X-box-binding protein 1* (*XBP1*). This splicing and re-ligation results in a translational frameshift to produce the active XBP1 protein, a basic leucine zipper (bZIP) family transcription factor that can bind to both the ER stress response element (ERSE) and the unfolded protein response promoter elements (UPRE), thus transcriptionally up-regulating a number of genes involved in the UPR (Lee, et al., 2003). In addition, IRE1α is also necessary for cleavage and post-transcriptional degradation of many other mRNAs encoding secreted proteins. This plays a part in reducing protein loading onto the ER (Hollien & Weissman, 2006).

As a protein kinase, IRE1α can form protein complexes with TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1). This causes activation of ASK1 that in turn activates Jun N-terminal kinase (JNK) (Nishitoh, et al., 2002; Urano, et al., 2000). Moreover, binding of IRE1α to TRAF2 leads to activation of a number of other protein kinases implicated in immunity and inflammation (Urano, et al., 2000). IRE1α can also bind to the multi-domain pro-apoptotic Bcl-2 family proteins Bax and Bak (Hetz, et al., 2006). This association may cause activation of IRE1α, and thus modulating calcium flux and UPR signalling.

2.2 ATF6

ATF6 has two isoforms, α and β (Ma & Hendershot, 2004; Ron & Walter, 2007; Schröder & Kaufman, 2005). They are members of the bZIP transcription factor family and have conserved protein domain, but interestingly, divergent transcriptional activation domains. ATF6α is the better characterized of the two that shares many of the activities of IRE1α. Upon ER stress it is freed from the ER membrane and translocates to the Golgi compartment, where it is cleaved by the S1P/S2P serine protease to produce a transcription factor that binds to ERSE elements, thus activating genes encoding proteins involved in the UPR including XBP1, C/EBP homologous protein (CHOP), and ER chaperone proteins such as GRP78 and GRP94 (Okada, et al., 2002; Yoshida, et al., 2001).

2.3 PERK

PERK is a serine/threonine protein kinase. Oligomerization of PERK at the ER membrane leads to its autophosphorylation and activation (Ma & Hendershot, 2004; Ron & Walter, 2007; Schröder & Kaufman, 2005). Activated PERK phosphorylates the translation initiation factor, eukaryotic initiation factor 2 α (eIF2α), leading to its inactivation and attenuation of translation. However, selective mRNAs can be preferentially translated. The best characterized of these is the transcription factor ATF4 that activates the transcription of genes including CHOP, GRP78, and GRP94. PERK$^{-/-}$ cells and cells expressing eIF2α that cannot be phosphorylated are hypersensitive to ER stress-induced cell death, indicating the importance of signals initiated by PERK in protection of cells from ER stress (Harding, et al., 2000).

3. ER stress and cell death

Although ER stress activates the UPR that is essentially a cyto-protective response, prolonged or excessive activation of the UPR can result in cell death by inducing primarily apoptosis (Ma & Hendershot, 2004; Ron & Walter, 2007; Schröder & Kaufman, 2005). In some circumstances, ER stress can also trigger autophagy that contributes to degradation of excessive proteins and protects cells from apoptosis (Høyer-Hansen & Jäättelä, 2007), but similar to the UPR, excessive autophagy can lead to autophagic cell death (Codogno & Meijer, 2005). Moreover, ER stress has been shown to play a role in oncogene-induced senescence (Denoyelle, et al., 2006).

Induction of apoptosis by ER stress in most cell types involves many of the same molecules that have important roles in other apoptotic cascades including Bcl-2 family proteins and caspases (Boyce & Yuan, 2006; Ferri & Kroemer, 2001). However, how ER stress-induced apoptotic signalling is triggered may vary among different cell types and ER stress inducers in question.

3.1 The mitochondrial apoptotic pathway and ER stress-induced apoptosis

Involvement of the mitochondrial apoptotic pathway in ER stress-induced apoptosis has been well-documented (Boyce & Yuan, 2006; Ferri & Kroemer, 2001), although there have also been reports showing that, at least in some circumstances, ER stress can trigger apoptosis independently of mechanisms mediated by mitochondria (Rao, et al., 2002).

The mitochondrial apoptotic pathway is tightly regulated by interactions between pro- and anti-apoptotic Bcl-2 family proteins (Cory & Adams, 2002). Among them, anti-apoptotic

proteins such as Bcl-2, Bcl-X_L, and Mcl-1 protect mitochondrial integrity, whereas pro-apoptotic members of the family promote the release of apoptogenic proteins such as cytochrome c, second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis-binding protein with low pI (DIABLO) from mitochondria (Du, et al., 2000; Li, et al., 1997; Verhagen, et al., 2000). The pro-apoptotic proteins of the Bcl-2 family can be further divided into BH3-only proteins such as Bim, PUMA, and Noxa and their effectors, the multidomain proteins Bax and Bak. Activation of BH3-only proteins are essential in induction of apoptosis, as they act as "death ligands" to activate Bax and Bak by directly binding to them, or by indirectly displacing them from anti-apoptotic Bcl-2 family members. The interactions between BH3-only proteins and anti-apoptotic proteins are highly selective (Chen, et al., 2005). For example, while Bim and PUMA can tightly engage all the anti-apoptotic proteins, thus being particularly potent apoptosis initiator, Bad can only bind to Bcl-2 and Bcl-X_L, but not Mcl-1. In addition, Bid and Bim have been shown to be the only BH3-only proteins that can directly bind to and activate Bax (Kuwana, et al., 2005). Anti-apoptotic proteins of the family may also constitute more than one functional class. For instance, Mcl-1 was proposed to play a unique apical role, elimination of which is required at an early stage of induction of apoptosis (Nijhawan, et al., 2003).

The multidomain pro-apoptotic Bcl-2 family proteins Bax and Bak can localize to the ER membrane, where they bind to IRE1α, which has been shown to be required for activation of IRE1α and subsequent activation of XBP1 (Zong, et al., 2003). Loss of Bax/Bak leads to, at least in some cases, impairment of adaptation to ER stress (Hetz, et al., 2006). It appears therefore that Bax/Bak may have a pro-survival role in the ER stress response, presumably due to activation of the IRE1α/XBP-1 pathway. Indeed, ER localized Bax/Bak has been suggested to act as an adaptive mechanism under conditions of mild or transient ER stress, but as pro-apoptotic molecules under prolonged or strong ER stress conditions (Heath-Engel, et al., 2008; Hetz & Glimcher, 2008).

Involvement of Bax- and Bak-dependent mitochondrial apoptotic pathway strongly suggests that one or more BH3-only proteins play critical roles in ER stress-induced apoptosis. This is further supported by inhibition of ER stress-induced apoptosis with over-expression of Bcl-2 or one of its anti-apoptotic homologs (Boyce & Yuan, 2006; Ferri & Kroemer, 2001). Indeed, ER stress can up-regulate PUMA and Noxa in various types of cells (Armstrong, et al., 2007; Li, et al., 2006; Wang, et al., 2009). This has been shown to be p53-dependent or –independent presumably related to different types of cells used in varying studies and ER stress inducers in question. For example, p53-dependent up-regulation of PUMA and Noxa has been demonstrated in mouse embryo fibroblasts exposed to tunicamycin and thapsigargin (Li, et al., 2006), which are commonly used laboratory tools as ER stress inducers. Tunicamycin induces ER stress by inhibition of glycosylation, whereas thapsigargin, by inhibition of ER Ca^{2+} ATPases. In contrast, p53-independent activation of PUMA was observed in human osteosarcoma SAOS-2 cells, and colon cancer HCT116 cells subjected to ER stress (Reimertz, et al., 2003).

Another BH3-only protein that plays an important role in ER stress-induced apoptosis in certain circumstances is Bim. This was initially suggested by translocation of Bim to the ER upon induction of ER stress, and was further supported by the finding that ectopic expression of ER-targeted Bim induced apoptosis (Morishima, et al., 2004). More recently, Bim was found to be transcriptionally up-regulated in diverse types of cells under stress (Puthalakath, et al., 2007). This is mediated by binding of heterodimers of the transcription

factors CHOP and C/EBPα to an unconventional promoter within the first intron of the Bim gene. In addition, ER stress triggers protein phosphatase 2A-mediated dephosphorylation of Bim, which prevents its ubiquitination and proteasomal degradation.

The BH3-only protein BIK is predominantly located to the ER, where it forms complex with GRP78. BIK has been shown to regulate Bax- and Bak-dependent release of calcium from the ER and mitochondrion-mediated apoptosis in cells under ER stress (Fu, et al., 2007; Mathai, et al., 2005). Another BH3-only protein that has been reported to be activated by ER stress is Bad, which is under physiological conditions phosphorylated and sequestered in the cytoplasm by the protein 14-3-3, but was dephosphorylated in response to several ER stress stimuli (Elyaman, et al., 2002; Szegezdi, et al., 2008).

Besides up-regulation/activation of pro-apoptotic Bcl-2 family proteins, down-regulation of anti-apoptotic Bcl-2 family members by ER stress also contribute to ER stress-induced apoptosis. Transcriptional repression of Bcl-2 by CHOP has long been reported (McCullough, et al., 2001), but this is not universally observed in all cell type (Puthalakath, et al., 2007). Induction of ER stress may also impinge on Bcl-2 phosphorylation and stability. This has been suggested to be mediated by protein phosphatase 2A (Lin, et al., 2006). Down-regulation of Mcl-1 has also been frequently observed. This is, at least in some cases, due to inhibition Mcl-1 mRNA translation (Fritsch, et al., 2007).

3.2 Caspases and ER stress-induced apoptosis

Activation of initiator caspases is a proximal event in induction of apoptosis by either the intrinsic (mitochondrial) or extrinsic (death receptor) apoptotic pathways. Although processing of caspase-8, -9, and -2 has been observed in cells under ER stress, which eventually leads to activation of effector caspases such as caspase-3 and -7, activation of caspase-12 in rodents and caspase-4 in human appears essential in ER stress-induced apoptosis (Ferri & Kroemer, 2001; Hitomi, et al., 2004).

Caspase-12 is located to the ER and is selectively activated by ER stress (Hitomi, et al., 2004). Caspase-12 deletion inhibits apoptosis induced by a variety ER stress inducers such as tunicamycin and thapsigargin. In contrast, over-expression of caspase-12 induces apoptosis. There is large body of evidence showing that caspase-12 is activated by ER stress up-stream of effector caspases, indicating it functions as an initiator caspase. However, caspase-12 is expressed only in rodents. Its human homologue is silenced by several mutations during evolution (Fischer, et al., 2002). Human caspase-4 is the closest homologue to murine caspase-12 and is at least partially located to the ER (Hitomi, et al., 2004). Caspase-4 has been shown to fulfil the function of caspase-12 in ER stress-induced apoptosis in some types of human cells (Hitomi, et al., 2004; Jiang, et al., 2007).

How caspase-12 and caspase-4 is activated in cells under ER stress remains to be determined. A number of biologic events have been shown to be associated with activation of caspase-12 in murine cells (Boyce & Yuan, 2006; Heath-Engel, et al., 2008; Nakagawa, et al., 2000; Urano, et al., 2000). First, caspase-12 activation has been linked to release of calcium from ER and consequent activation of calpain. Inhibition of calpain by chemical inhibitors and genetic approaches and chelation of intracellular calcium can block caspase-12 activation induced by various ER stress inducers; second, ER stress triggers recruitment of caspase-7 to the ER where it complexes with caspase-12 leading to its activation. GRP78 also exists at the complex and plays a role in inhibiting both caspase-7 and -12; and third, it has been suggested that caspase-12 is associated with TRAF2, but under ER stress,

recruitment of the TRAF2/caspase-12 complex to IRE1α provides a scaffold for caspase-12 activation. However, if human caspase-4 is similarly activated as murine caspase-12 by ER stress remains to be clarified.

4. Adaptation of melanoma cells to ER stress

Although excessive or prolonged UPR can result in apoptosis, most melanoma cell lines are not sensitive to apoptosis induced by pharmacological ER stress inducers, tunicamycin and thapsigargin. This suggests that melanoma cells may have adapted to ER stress conditions by development of resistance mechanisms against ER stress-induced apoptosis.

There is ample evidence showing that the UPR is activated in various solid tumours due to both intrinsic and extrinsic factors (Lee, 2007; Ma & Hendershot, 2004). Increased expression of GRP78, a commonly used indicator of activation of the UPR, has been reported in a variety of cancers. In some cases, GRP78 expression is associated with tumour growth and resistance to chemotherapy (Lee, 2007; Ma & Hendershot, 2004). Consistently, GRP78 is also expressed at varying, but commonly higher levels in melanoma cells relative to melanocytes both *in vitro* and *in vivo* (Jiang, et al., 2009a; Zhuang, et al., 2009). In addition, the levels of GRP78 correlate with progression of the disease and other markers of prognosis such as tumour thickness and mitotic rate (Zhuang, et al., 2009). Similarly, the active form of XBP1 mRNA is expressed at higher levels in cultured melanoma cells (Jiang, et al., 2009a). Therefore, melanoma cells have adapted to ER stress, which appears to be imposed on melanoma cells at early stages of development, in that UPR has been shown to be activated at initiation stages of melanoma by the oncogenic form of HRAS ($HRAS^{G12V}$) (Denoyelle, et al., 2006).

It is conceivable that cells in a developing tumour without sufficient blood supply may undergo hypoxia, nutrient starvation and acidosis (Lee, 2007; Ma & Hendershot, 2004). In addition, mutated proteins in cancer cells may also contribute to ER stress. Another ER stress-inducing mechanism of cancer cells is increased glycolytic activity due to the Warburg effect. High levels of lactate dehydrogenase (LDH) are readily apparent in patients with melanoma, and the levels in sera are the single most powerful predictor of prognosis in metastatic disease (Balch, et al., 2009; Eton, et al., 1998). In support of the importance of increased glycolysis in induction of ER stress in melanoma, the levels of LDH5 are correlated with the levels of GRP78 in melanoma cells *in vivo* (Zhuang, et al., 2010).

5. Adaptive mechanisms of melanoma cells to ER stress

Adaptation to ER stress is believed to be an intrinsic consequence of low level activation of the UPR. However, it remains a paradox how the UPR switches between the pro-survival and pro-apoptotic signalling pathways. Nevertheless, adaptive processes converge on mechanisms that inhibit apoptosis. Because ER stress can induce apoptosis through multiple mechanisms, it is conceivable that adaptation of melanoma cells to ER stress is the consequence of activation of multiple anti-apoptotic mechanisms.

5.1 Up-regulation of Mcl-1 is critical for survival of melanoma cells upon ER stress

Mcl-1 is an anti-apoptotic Bcl-2 family protein that is of particular importance in melanoma, in that its expression increases with melanoma progression and is associated with poor

prognosis (Zhuang, et al., 2007). Moreover, Mcl-1 is a major resistance mechanism of melanoma cells to apoptosis induced by a variety of apoptotic stimuli (Jiang, et al., 2008; Wang, et al., 2007). As a protein with a rapid turn-over rate, Mcl-1 expression is frequently regulated by post-translational mechanisms (Schwickart, et al., 2010; Warr & Shore, 2008; Zhong, et al., 2005). Nevertheless, an increase in the Mcl-1 protein levels often correlates with an increase in its mRNA levels, mostly due to enhanced transcription (Iglesias-Serret, et al., 2003; Warr & Shore, 2008). In addition, Mcl-1 can be regulated at the translational level. For example, the Mcl-1 protein levels have been shown to be markedly reduced by thapsigargin due to translational repression mediated by phosphorylation of eIF2α downstream of PERK (Fritsch, et al., 2007).

In contrast to down-regulation by ER stress in many other cell types, Mcl-1 along with Bcl-2 is up-regulated in melanoma cells by ER stress (Jiang, et al., 2008). Although up-regulated, Bcl-2 does not appear to be critical for protection of melanoma cells from ER stress-induced apoptosis, in that inhibition of Bcl-2 by siRNA has only a minimal effect on sensitivity of melanoma cells to ER stress-induced apoptosis, whereas over-expression of Bcl-2 can only delay the onset of apoptosis, but does not rescue melanoma cells from apoptosis induced by ER stress when Mcl-1 is deficient. On the other hand, siRNA inhibition of Mcl-1 readily enhances ER stress-induced apoptosis, and over-expression of Mcl-1 efficiently protects melanoma cells from apoptosis induced by tunicamycin or thapsigargin, even when Bcl-2 is inhibited. Therefore, Mcl-1, but not Bcl-2, plays a determining role in survival of melanoma cells under ER stress conditions. Indeed, while the Mcl-1 expression is associated with melanoma progression, the expression of Bcl-2 decreases during progression of melanoma (Zhuang, et al., 2007). Importantly, the levels of Mcl-1 in melanoma cells are correlated with the levels of GRP78, suggesting that the increase in Mcl-1 in melanoma may be a consequence of activation of the UPR and an adaptive mechanism of melanoma cells to ER stress (Zhuang, et al., 2009).

Up-regulation of the Mcl-1 protein in melanoma cells under ER stress is associated with an increase in the Mcl-1 mRNA, which is efficiently inhibited by actinomycin D, a general transcription inhibitor, indicating that a transcriptional increase is involved in ER stress-induced up-regulation of Mcl-1 (Jiang, et al., 2008). Analysis of the Mcl-1 promoter region identifies a binding site for the E26 transformation specific sequence (Ets)-1 that is activated by treatment with tunicamycin or thapsigargin (Dong, et al., 2011). Ets-1 is a member of the Ets family of transcription factors that play roles in many biologic processes such as cell growth and survival (Dittmer, 2003; Hahne, et al., 2008). Indeed, inhibition of Ets-1 by siRNA or mutations in the Ets-1 binding site blocks up-regulation of the Mcl-1 transcript by ER stress, and recapitulates the effect of inhibition of Mcl-1 on sensitization of melanoma cells to ER stress-induced apoptosis (Dong, et al., 2011). Therefore, Ets-1 is responsible for transcriptional up-regulation of Mcl-1 by ER stress in melanoma cells.

Ets-1 is expressed at high levels in many types of cancers and is involved in many biological processes in cancer cells such as cell growth and survival (Davidson, et al., 2001; Span, et al., 2002). In melanoma, the levels Ets-1 were similarly found to be higher than those in benign melanocytic lesions and melanocytes and to increase with progression of the disease (Rothhammer, et al., 2004; Torlakovic, et al., 2004). Although the role of Ets-1 in regulation of apoptosis may vary between different cell types, transcriptional up-regulation of Mcl-1 by Ets-1 in melanoma cells subjected to ER stress indicates that it is critical in protection of melanoma cells against ER stress-induced apoptosis (Dong, et al., 2011).

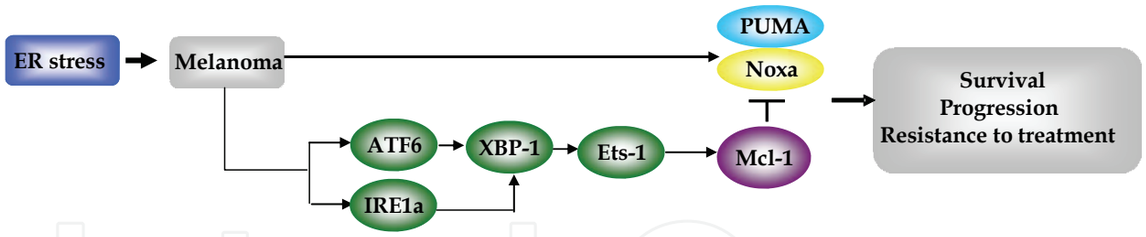

Fig. 2. A schematic illustration of Ets-1-mediated up-regulation of Mcl-1 downstream of XBP-1 in melanoma cells under ER stress

Importantly, Ets-1 is also transcriptionally up-regulated in melanoma cells upon induction of ER stress, which is partially inhibited in melanoma cells deficient in IRE1α or ATF6, indicating that these branches of the UPR are involved in the ER stress-triggered increase in Ets-1 in melanoma cells (Dong, et al., 2011). The convergence of the IRE1α and ATF6 pathways on XBP-1 suggest that XBP-1 may be involved in up-regulation of Ets-1 by the UPR. Indeed, inhibition of XBP-1 blocks ER stress-induced up-regulation of Ets-1 (Dong, et al., 2011). Thus, XBP-1 plays an important role in up-regulation of Ets-1, and subsequent up-regulation of Mcl-1 in melanoma cells subjected to ER stress.

Taken together, it appears that a signalling nodule of XBP1-Ets-1-Mcl-1 leading to transcriptional up-regulation of Mcl-1 is activated in melanoma cells upon activation of the UPR, which is important for protection of melanoma cells against ER stress-induced apoptosis by antagonizing pro-apoptotic Bcl-2 family proteins, such as PUMA and Noxa that are also increased by ER stress in melanoma cells (Fig. 2) (Jiang, et al., 2008).

5.2 Induction of GRP78 in adaptation of melanoma cells to ER stress

As the "master regulator" of the response of cells to ER stress, GRP78 plays an important role in survival of cells under ER stress (Lee, 2005, 2007). Multiple mechanisms have been reported to contribute to GRP78-mediated inhibition of apoptosis in cell under ER stress. These include its binding to unfolded/misfolded proteins to limit their aggregation, binding to calcium to maintain calcium homeostasis, and binding to caspase-12 and caspase-7 to inhibit their activity (Lee, 2005, 2007). In addition, GRP78 has been shown to promote cell proliferation and to be necessary for ER stress-induced autohpagy (Li, et al., 2008). Although the three branches of the UPR can all contribute to induction of GRP78, the ATF6 pathway plays a dominant role in regulation of GRP78 expression (Harding, et al., 2002; Zhang & Kaufman, 2004).

As a typical ER lumenal chaperone, GRP78 can also be expressed on the surface of various types of cancer cells including melanoma cells, in particular, when cells are under ER stress (Gonzalez-Gronow, et al., 2009; Lee, 2007). Relocation of GRP78 to the cell surface appears to be mediated its C-terminal ER retention motif, as deletion of the motif alters its cell surface presentation (Gonzalez-Gronow, et al., 2009). Cell surface GRP78 interacts with a number of cell surface proteins and soluble ligands such as activated α(2)-macroglobulin and acts as an initiator of intracellular signalling pathways, thus promoting cell survival and proliferation (Gonzalez-Gronow, et al., 2009; Zhang, et al., 2010).

Autoantibodies that react with GRP78 expressed on the cell surface can be detected in the sera of patients with prostate cancer, ovarian cancer and melanoma (Gonzalez-Gronow, et al., 2009). These autoantibodies are a negative prognostic factor in prostate cancer (Gonzalez-Gronow, et al., 2009). However, if they are of prognostic value in melanoma

patients has not been established. Nevertheless, binding of autoantibodies to the cell surface GRP78 has been reported to promote tumour growth of a murine melanoma model (de Ridder, et al., 2010). Because its preferential expression on the cell surface of cancer cells, GRP78 has been suggested to be a tumour-associated antigen (Misra, et al., 2011).

5.3 Sustained IRE1α signalling may be essential for adaptation of melanoma cells to ER stress

No trigger for ER stress has been identified that selectively elicits only protective responses or apoptosis. However, the duration of activation of individual arms of the UPR plays an important role in determining cell fate in response to ER stress. The IRE1 pathway is rapidly attenuated after induction of ER stress even in the presence of ER stress inducers, whereas the ATF6 branch is also attenuated, albeit with slow kinetics. In contrast, the PERK pathway of the UPR persists for considerably longer periods, and is presumably responsible for induction of apoptosis in the absence of activation of IRE1 and ATF6 (Lin, et al., 2007; Rubio, et al., 2011). It appears that cyto-protective outputs of the initial combined activation of three arms of the UPR outweigh pro-apoptotic outputs. However, attenuation of IRE1 and ATF6 signalling create an imbalance that leads to apoptosis. Consistent with this, IRE1α - or ATF6-deficient cells demonstrate reduced survival rate in cells treated with ER stress inducers.

In agreement with this model, deficiency in IRE1α- or ATF6- renders melanoma cells sensitivity to apoptosis induced by ER stress. In contrast, melanoma cells deficient in PERK remain relatively resistant to ER stress-induced apoptosis (Hersey & Zhang, 2008). It has been found that elevated levels of GRP78 along with phosphorylated PERK and eIF2a, persisted for at least 36 hours in the presence tunicamycin and thapsigargin (Hersey & Zhang, 2008). This suggests that, in contrast to observations made in other cell types, IRE1 and ATF6 signalling in melanoma cells was not rapidly attenuated under prolonged ER stress. Therefore, a testable hypothesis is that perpetuation of IRE1 and/or ATF6 signalling with or without attenuation of the PERK pathway is an essential mechanism of adaptation of melanoma cells to ER stress.

How the duration of activation of the UPR pathways is regulated is not entirely clear, although it is known that negative feed-back mechanism exists to switch off UPR signalling once stress imposed on the ER is resolved (Zhang & Kaufman, 2004). Recently, it was found that rapid attenuation of the IRE1/XBP1 pathway is associated with binding of IRE1α with Bax inhibitor-1 (BI-1), an evolutionarily conserved ER-resident protein (Bailly-Maitre, et al., 2010; Lisbona, et al., 2009).

BI-1 was initially identified as an inhibitor of Bax-induced apoptosis that is a transmembrane protein functionally related to the BCL-2 family of proteins and is primarily located to the ER membrane (Xu & Reed, 1998). Although BI-1 has no obvious homology with Bcl-2-related proteins, it physically interacts with different members of this family such as BCL-2 and Bcl-X_L (Xu & Reed, 1998). BI-1 has been known to protect cells from apoptosis induced by various stimuli, including ER stress (Robinson, et al., 2011). However, BI-1 deficient cells displays hyperactivation of IRE1α, leading to increased levels of activation of XBP-1 and upregulation of UPR target genes (Lisbona, et al., 2009). This is associated with the formation of a stable protein complex between BI-1 and IRE1α, decreasing its ribonuclease activity. If regulation of IRE1α by BI-1 is related to BAX and Bak that can also bind to IRE1α on the ER membrane is currently unclear, it is known however that the ER-associated RING-type E3 ligase bifunctional apoptosis regulator (BAR) interacts with BI-1 and promotes its proteasomal degradation (Rong, et al., 2011).

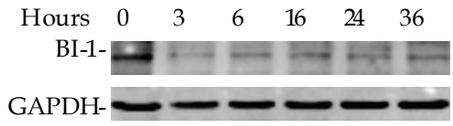

Fig. 3. ER stress down-regulates BI-1 in melanoma cells. Whole cell lysates from a melanoma cell line treated with TM (3μM) for indicated periods were subjected to Western blot analysis of BI-1 and GAPDH (as a loading control)

Interestingly, sustained activation of the IRE1/XBP1 pathway in melanoma cells upon induction of ER stress is associated with a decrease in the expression of BI-1 (Fig. 3), but whether there is a cause and effect relationship between two events remains to be studied. In addition, if down-regulation of BI-1 by ER stress is due to by enhancement in BAR-mediated proteasomal degradation is currently unknown.

5.4 Dysregulation of the CHOP/Bim axis in adaptation of melanoma cells to ER stress

CCAAT/enhancer binding protein homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) was first identified as a DNA-damage-inducible gene of the C/EBP family of transcription factors (Ron & Habener, 1992). CHOP is ubiquitously expressed at low levels, but its expression can be induced by various cellular stress conditions, such as DNA damage and nutrient depletion (Ron & Habener, 1992). Induction of CHOP has been shown to be essential for ER stress-induced apoptosis, in that CHOP$^{-/-}$ mice exhibit reduced apoptosis in response to ER stress (Oyadomari, et al., 2002; Oyadomari & Mori, 2004). Although the three pathways of the UPR can all mediate transcriptional up-regulation of CHOP, the PERK branch appears to play a dominant role in induction of CHOP in cells under ER stress (Oyadomari & Mori, 2004).

CHOP-induced apoptosis was initially shown to be associated with transcriptional repression of Bcl-2 expression (McCullough, et al., 2001). Up-regulation of the BH3-only proteins PUMA and Noxa in cells under ER stress has also been reported to be associated with CHOP (Li, et al., 2006). More recently, it was demonstrated that CHOP can transcriptionally up-regulate another BH3-only protein Bim in diverse types of cells (Puthalakath, et al., 2007). This requires formation of CHOP-C/EBPα heterodimers, which then bind to a non-conventional promoter within the first intron of the *Bim* gene. Inhibition of phosphorylation of Bim by protein phosphatase 2A (PP2A) also contributes the increased Bim expression in cells subjected to ER stress. Dephosphorylation of Bim by PP2A enhances the stability of the Bim protein. In addition, CHOP can up-regulate the death receptor, TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2), thus activating the death receptor pathway enhancing TRAIL-induced apoptosis in many cell types (Lin, et al., 2008; Yamaguchi & Wang, 2004).

Nevertheless, the CHOP/Bim axis-mediated apoptosis signalling does not appear to function effectively in melanoma cells, even though CHOP is strongly induced by ER stress along with activation of PERK and phosphorylation of eIF2α in the cells under ER stress (Fig. 4). Strikingly, while over-expression of CHOP does not confer sensitivity of melanoma cells to ER stress-induced apoptosis, inhibition of CHOP by siRNA resulted in low levels of apoptosis in the cells (Zhang, et al. unpublished data). These results suggest that, instead inducing apoptosis, CHOP may have a pro-survival role in melanoma cells, as it does in neurons (Halterman, et al., 2010). How the biological function of CHOP is switched from pro-apoptotic to pro-survival in melanoma cells remains, however, to be elucidated.

Induction of ER stress causes up-regulation of Bim mRNA in melanoma cells. However, the increase in Bim expression at the protein level appears to be transient, which is associated with an increase in phosphorylation of the protein (Fig. 4), in contrast to its dephosphorylation by ER stress in many other cell types. Increased phosphorylation of Bim is, at least in part, due to activation of the MEK/ERK pathway, in that inhibition of MEK by the small molecule inhibitor U0126 blocked Bim phosphorylation and increased the Bim expression at the protein level in melanoma cells subjected to tunicamycin (Zhang et al. unpublished data). The MEK/ERK pathway is known to be constitutively activated in melanoma cells (Jiang, et al., 2007). It is therefore conceivable that phosphorylation of Bim by ERK is dominant over its dephosphorylation by PP2A in melanoma cells when subjected to ER stress, thus leading to its rapid degradation by the proteasome system. Although the exact mechanism needs to be further clarified, dysregulation of the CHOP/Bim axis appears to play an important role in survival of melanoma cells under ER stress.

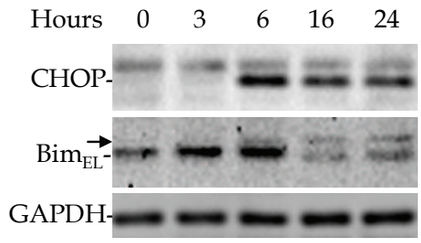

Fig. 4. ER stress induces changes in the expression CHOP and Bim in melanoma cells. Whole cell lysates from a melanoma cell line treated with TM (3μM) for indicated periods were subjected to Western blot analysis. The arrow heads points to bands of phosphorylated Bim_{EL}

Some other mechanisms that are involved in protection of melanoma cells from ER stress-induced apoptosis include activation of the PI3K/Akt signalling pathway and induction of the apoptosis repressor with caspase recruitment domain (ARC) protein (Chen, et al., 2008; Jiang, et al., 2009c). The list is expected to increase with better understanding of the mechanisms of induction of apoptosis by ER stress. Therefore, targeting multiple adaptive mechanisms may be needed to overcome resistance of melanoma cells to ER stress-induced apoptosis.

6. Adaptation to ER stress as a resistance mechanism of melanoma cells chemotherapeutic drugs

A growing body of evidence shows that induction of ER stress and subsequent activation of the UPR can alter chemosensitivity of cancer cells (Hersey & Zhang, 2008; Lee, 2007; Ma & Hendershot, 2004). On the other hand, induction of apoptosis is associated with induction of ER stress in many types of cancer cells by a number of different classes of chemotherapeutic drugs, such as the DNA-damaging agent cisplatin, the non-steroidal anti-inflammatory drug celecoxib, the proteasomal inhibitor bortezomib, and the general kinase inhibitor sorafenib, suggesting that induction of ER stress may be an important mechanism in killing of cancer cells by chemotherapeutic drugs (Hersey & Zhang, 2008; Hill, et al., 2009; Jiang, et al., 2009c; Lee, 2007; Ma & Hendershot, 2004; Martin, et al., 2010). On the other hand, melanoma cells have developed multiple adaptive mechanisms that render the cells largely resistant to ER stress-induced apoptosis, suggesting that adaptation to ER stress may contribute to

resistance of melanoma cells to induction of apoptosis (Hersey & Zhang, 2008). Some adaptive mechanisms to ER stress, such as induction of GRP78 and Mcl-1, have been shown to play important roles in resistance of melanoma cells to various chemotherapeutic drugs (Hersey & Zhang, 2008; Jiang, et al., 2009a; Jiang, et al., 2009c).

6.1 GRP78 contributes to resistance of melanoma cells to cisplatin and adriamycin

Although cisplatin and adriamycin are conventionally regarded as DNA-damaging agents, they can induce ER stress in melanoma cells as shown by increased expression of GRP78 and activation of XBP-1 (Jiang, et al., 2009c). Whether induction of ER stress plays a part in induction of apoptosis by the drugs remains to be clarified, but GRP78 protects melanoma cells against cytotoxic effects of the drugs (Jiang, et al., 2009a). This is mediated, at least in part, by the inhibitory effect of GRP78 on activation of caspase-4. The latter is bound to and kept inactive by GRP78 in melanoma cells (Jiang, et al., 2007; Jiang, et al., 2009a). Inhibition of GRP78 by siRNA in melanoma cells subjected to ER stress frees caspase-4 and leads to its activation (Jiang, et al., 2007).

There was no correlation between the GRP78 expression levels and sensitivities of melanoma cell lines to cisplatin or adriamycin, suggesting that, besides GRP78, other mechanisms may also contribute to regulation of responses of melanoma cells to the drugs (Jiang, et al., 2009a). For example, expression of ATP-binding cassette (ABC) transporters and increased DNA repair are known to contribute to resistance of cancer cells against cisplatin and adriamycin (Frank, et al., 2005; Hall, et al., 2008). In addition, activation survival signalling pathways such as the PI3K/Akt and MEK/ERK pathways is a common cause for resistance of melanoma to apoptosis (Hersey, et al., 2006; Soengas & Lowe, 2003). Regardless, targeting GRP78 may be a useful strategy in sensitizing melanoma cells to these chemotherapeutic drugs.

6.2 Melanoma cells under ER stress are more resistance to microtubule-targeting durgs

Unlike cisplatin and adriamycin, the microtubule-targeting drugs docetaxel and vincristine do not trigger ER stress in melanoma cells as shown by their inability to induce GRP78 and the spliced XBP-1 mRNA. However, their cytotoxicity is attenuated in melanoma cells subjected to ER stress (Jiang, et al., 2009c). This was demonstrated by the observation that treatment with the ER stress inducer tunicamycin or thapsigargin before the addition of docetaxel or vincristine reduced the levels of apoptosis induced by the drugs. GRP78 does not appear to be involved in that sensitivity of melanoma cells to docetaxel- and vincristine-induced apoptosis cannot be enhanced by inhibition of GRP78. In contrast, activation of the PI3k/Akt pathway downstream of XBP-1-mediated signalling is critical in protection of melanoma cells against the drugs by preloaded ER stress (Jiang, et al., 2009c).

How Akt is activated by XBP-1 signalling in melanoma cells remains to be further studied, but it was recently shown that ER stress activated Akt in a zebrafish embryonic cell line through XBP-1-mediated up-regulation of insulin growth factor-1 (IGF-1) (Hu, et al., 2007). However, IGF-1/IGF-1 receptor signalling in melanoma cells originates mainly from exogenous IGF-1 because melanoma cells express no, or minimal, IGF-1 (Lee, et al., 2008; Rodeck, et al., 1991). Nevertheless, it is possible that XBP-1 may activate a factor or factors similar to IGF-1 that in turn causes activation of the PI3K/Akt pathway in melanoma cells under ER stress. In any case, it appears that activation of XBP-1 signalling as a consequence of adaptation to ER stress is an important resistance mechanism of melanoma cells to the microtubule-targeting drugs docetaxel and vincristine.

6.3 Melanoma cells under ER stress are more susceptible to apoptosis induced by the BH3 mimetic obatoclax

Small molecule mimics of BH3-only proteins are emerging as promising anti-cancer agents by inhibiting anti-apoptotic Bcl-2 family proteins. Obatoclax, also known as GX015-070, was identified by chemical library screening to bind the hydrophobic groove of anti-apoptotic Bcl-2 family proteins and antagonize their function (Nguyen, et al., 2007). Obatoclax can efficiently neutralize all anti-apoptotic Bcl-2 family proteins including Mcl-1, and has been reported to potently kill some types of cancer cells, such as myeloma cells, as a single agent, and to enhance apoptosis induced by various apoptotic stimuli such as TNF-related apoptosis inducing ligand (TRAIL) and the proteasome inhibitor bortezomib (Huang, et al., 2009; Konopleva, et al., 2008).

Because up-regulation of Mcl-1 is an important adaptive mechanism of melanoma cells to ER stress, it is conceivable that obatoclax may interrupt the adaptation by antagonizing Mcl-1. Indeed, obatoclax has been shown to potently overcome resistance of melanoma cells to apoptosis induced by the ER stress inducers tunicamycin and thapsigargin (Jiang, et al., 2009b). Besides its inhibitory effect on Mcl-1, obatoclax triggers further increases in the levels of the BH3-only protein Noxa in melanoma cells undergoing ER stress, which in turn cooperates with obatoclax in activating the mitochondrial apoptotic pathway (Jiang, et al., 2009b). Therefore, obatoclax is a potent agent that targets a major adaptive mechanism to ER stress in melanoma. Therefore, combinations of obatoclax and agents that induce ER stress may be a useful strategy in the treatment of melanoma.

6.4 Targeting GRP78 to sensitize melanoma cells to apoptosis induced by Fenretinide and Bortezomib

Fenretinide is a synthetic retinoid deriverative that has been shown to induce ER stress and apoptosis in melanoma cells (Hill, et al., 2009; Lovat, et al., 2008). Bortezomib is a peptide boronate inhibitor of the proteasome that is similarly known to induce apoptosis in melanoma cells that is associated with induction of ER stress (Hill, et al., 2009; Qin, et al., 2005). Activation of the UPR appears to protect melanoma cells from apoptosis induced by the drugs as inhibition of GRP78 by either siRNA knockdown or a GRP78-specific subtilise toxin produces synergistic induction of apoptosis (Martin, et al., 2010). In addition, inhibition of protein disulfide isomerase (PDI), an ER enzyme that catalyzes the formation and breakage of disulfide bonds within proteins as they fold and plays a role in protecting cells from ER stress, similarly enhanced induction of apoptosis in melanoma cells by fenretinide and bortezomib (Lovat, et al., 2008). It is conceivable that inhibition of other adaptive mechanisms such as up-regulation of Mcl-1 may also sensitize melanoma cells to apoptosis induced by the drugs.

7. Conclusion

Primary events in the development of melanoma are gradually being pieced together (Bennett, 2008; Hersey, et al., 2011), but a more complete picture of evolution of the disease and resistance of metastatic melanoma to treatment requires additional understanding of secondary events consequent on initiation of the malignancy. In this chapter, we have provided evidence showing that an important driver of secondary events is signals resulting from adaptation of melanoma cells to ER stress, which are not only important for resistance of melanoma cells to ER stress-induced apoptosis, but also render melanoma cells resistant to various chemotherapeutic drugs.

Although targeting adaptive mechanisms such as GRP78 and Mcl-1 have been shown to sensitize melanoma cells to apoptosis induced by a number of chemotherapeutic drugs, its effects on efficacy of other promising therapeutic agents such as inhibitors against the RAF/MEK/ERK and PI3K pathways need to be evaluated (Hersey, et al., 2011). Cross-talks between the UPR and the pathways are conceivable to play a role in regulation of sensitivity of melanoma cells to the inhibitors.

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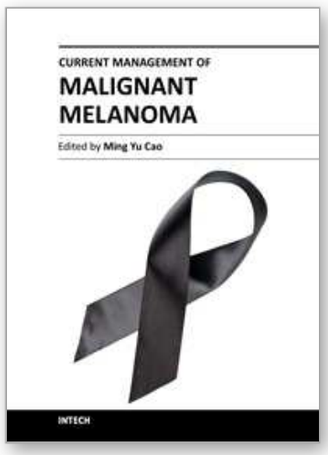

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Management of melanoma is challenging, especially for the late stage of the disease. Development of new therapies and optimizing current treatments are being pursued in attempt to further improve the survival rate. The book provides up-to-date knowledge and experience in early diagnosis, prevention and treatment of melanoma as well as current ongoing clinical studies on melanoma. The book also provides the most recent perspectives of research on the molecular basis of melanoma, such as melanoma associated genes and a possible link between stress and melanoma.

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