SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

# Mitochondrial expression of TASK-3 channels in human malignant melanoma cell cultures and their role in cell survival and proliferation

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# Introduction

TASK-3 channels belong to the most recently discoverer superfamily of $K^+$ channels whose members are dimeric channels with each subunit containing two pore forming regions. TASK-3 channels are involved in many physiological functions in mammalian cells: they have a role in the regulation of aldosterone secretion, the setting of neuronal excitability, and providing the hypoxia sensitivity of carotid body cell. They are also important in the modulation of neurotransmission in the central nervous system, as due to their pH sensitivity they can alter the postsynaptic effect of neurotransmitters.

While in the above mentioned functions TASK-3 channels exert their effect through the modulation of resting membrane potential, they are also involved in other processes where the exact mechanism of their effect is less clear. It must be mentioned that the activation of TASK-3 channels can induce apoptosis in certain cell types, while under different circumstances they seem to be involved in tumourigenesis. The amplification of the kcnk9 gene encoding the TASK-3 protein has been reported in the case of several cancer types, and the tumourigenic function of TASK-3 channels is also supported by experimental evidence. These results pointed out that not only the presence of the channel protein but also the $K^+$-permeable pore function is necessary for the tumourigenic effect. Although it is not known how exactly TASK-3 channels exert their effect promoting malignant cell proliferation, it is proposed that they increase cancer cells' tolerance against hypoxia and serum deprivation, which might be of great importance in the central, poorly vascularised regions of solid tumours. This assumption implies that TASK-3 channel function might somehow influence mitochondrial oxidation, though further examinations are needed to understand this connection.

The findings presented in this study not only provide evidence for the mitochondrial localisation of TASK-3 channels in cultured melanoma cells, but also prove that the presence

and function of TASK-3 channels might have crucial role in maintaining mitochondrial function, and thus cell survival under *in vitro* circumstances. These observations contribute to the understanding of the exact mechanism underlying the tumourigenic effect of TASK-3 channels, which might also have therapeutic implications.

# Overview of literature

## TASK-3 channels

TASK-3 channels belong to the superfamily of two pore domain (2P) $K^+$ channels. Regarding their subunit structure, functional properties and pharmacological characteristics, 2P $K^+$ channels significantly differ from the rest of potassium channels. In neurons 2P $K^+$ channels are involved in the setting of resting membrane potential, the regulation of input resistance and excitability of cells, and the modulation of action potential duration. The members of the TASK (TWIK-related acid-sensitive $K^+$-channels) family are characterised by their sensitivity to extracellular pH; their pore closes upon acidification.

The most typical expression site of human TASK-3 channels is the central nervous system, especially the cerebellum, where they are present in granule and Purkinje neurons as well as in astrocytes. Apart from this, they are also expressed in several other tissues, e.g. different parts of the gastrointestinal tract, pancreas and salivary gland ducts.

## TASK-3 channels: role in apoptosis

It is a very unusal phenomenon among potassium channels that TASK-3 channels are involved in the regulation of processes in connection with cell viability, such as apoptotic cell death. However, the data regarding TASK-3 channels' role in these processes are rather contradictory. While some studies support their pro-apoptotic role, it is also demonstrated that under different circumstances their function can prevent apoptotic cell death.

Neuronal excitability is a critical determinant of cell survival during brain development. As background potassium channels are responsible for both setting the resting membrane potential and determining action potential duration, the $K^+$ efflux resulting from their activation can be an important factor when it comes to cell survival or death. It is known that TASK-1/TASK-3 heterodimeric channels in the cerebellar granule cells of the rat play an important role in inducing apoptosis. During the development of the rat cerebellum, granule cells gradually move from the outer granule cell layer towards their final destination, while they significantly reduce in number due to apoptotic cell death. This phenomenon is of great physiological importance as much more granule cells are generated in the external granular layer than required for forming synapses with Purkinje cells, so the cell death induced by TASK current actively contributes to mathing the number of the two cell types. Presumably the mechanism underlying TASK channels' pro-apoptotic function is that the efflux of potassium ions is followed by water, which results in apoptotic volume decrease (AVD). Apart from this, the decrease of intracellular $K^+$ concentration can directly activate certain enzymes (e.g. caspases) involved in in the apoptotic process.

In contrast with the data mentioned above other studies emphasize that the hyperpolarisation caused by TASK channel function provides protection from cellular stress, and thus cell death. An interesting study pointed out that under hypoxia and serum deprivation TASK-3 channels can prevent apoptosis in both C8 murine fibroblast cells and cultured hippocampal slices.

## TASK-3 channels: role in tumorigenesis

The potential oncogenic function of TASK-3 channels was first proposed when the 3-10 fold amplification of the kcnk9 gene encoding the channel protein was described in 10% of malignant breast cancers, accompanied by 5-100 fold protein overexpression in 44% of the examined tumour samples. This overexpression showed no correlation with the

expression level of any established tumour markers (estrogen receptor, HER-2). Besides breast tumours, TASK-3 channel protein overexpression was described in malignant lung cancers as well.

Experimental evidence is also available supporting TASK-3 channels' role in promoting tumour formation. The expression of TASK-3 subunits in NMuMG mammalian epidermal cell line provided tumour-forming ability to cells when injected in mice. Furthermore it enhanced tumourigenicity of C8 murine embryonic fibroblasts as well as their resistance to hypoxia and serum deprivation. It was also observed that TASK-3 channels can contribute to the survival of tumour cells under in vitro circumstances, while their expression was described *in situ* in glioblastoma tumour samples as well, where their possible therapeutic significance was also proposed.

It is important to emphasize that according to experimental data the tumourigenic effect of TASK-3 channels is only exerted if they serve as functional $K^+$-permeable pores. Experiments comparing the functional, wild-type TASK-3 channel and its $K^+$-impermeable point mutant revealed that the oncogenic effect depends on the channel function. Immunosuppressed mice were inoculated with C8 cells expressing either the kcnk9 gene, or the G95E point mutant gene encoding a non-functional channel, and it was observed that the cells expressing the wild-type channels have a stonger tumourigenic potential. In order to examine the possible mechanism of the oncogenic effect, the wild-type and point mutant TASK-3 protein were expressed in embryonic murine fibroblast cells, and it was shown that the functional channel reduced the rate of TNF-induced apoptosis by 50% compared to the control, while the mutant channel protein was ineffective. This finding indicates that functional TASK-3 channels provide protection against TNF-induced apoptosis.

Apart from the theory that protection against apoptosis can promote malignant cell proliferation, other possibilities also have been proposed to explain the tumourigenic effect

of TASK-3 channels. Overexpression of TASK-3 channels might result in the hyperpolarization of the cell membrane, which increases the electrochemical gradient of calcium, causing $Ca^{2+}$-influx and thus alteration of cell proliferation and differentiation. Another possible explanation is that the $K^+$-efflux through TASK-3 channels in the cell surface membrane reduces the intracellular $K^+$-concentration, which can have a direct effect on the activity of certain enzymes (such as caspases). Tolerance against hypoxia and serum deprivation can also promote malignant cell proliferation, which might be especially important in the central, poorly vascularised area of solid tumours. Though it is unclear how exactly TASK-3 channels provide protection against hypoxia, this effect might be exerted not directly through the channel function, as hypoxia is known to inhibit TASK-3 channels. It is important to note that different substances inhibiting mitochondrial respiration have similar effect as hypoxia in the carotid body cells of the rat, reducing the amplitude of TASK-3 current through the cell surface membrane, so the activity of background potassium channels might be somehow related to the mitochondrial respiration processes.

## Aims of work

Our earlier findings revealed that melanoma cells maintained in cell culture show intensive TASK-3 immunopositivity with a dominantly intracellular pattern. This phenomenon raised several questions that we tried to answer in the frame of this study.

- In which intracellular organelle are TASK-3 channels expressed?
- Can the unusal expression pattern observed in melanoma cells be demonstrated in other, non-malignant cells, e.g. the TASK-3 positive HaCaT keratinocytes as well?
- How exactly TASK-3 channels contribute to maintaining viability of cultured melanoma cells, and what changes can be observed in the appearance and behaviour of cells when the synthesis of the channel protein is interfered with?

# Materials and methods

## Cell culturing, transfection

All cell lines were maintained in monolayer cell cultures at 37°C, 5% $CO_2$ content and 100% relative humidity. Melanoma cells were cultured in RPMI 1640 medium, while C2C12, HaCaT, and HEK293 cell were cultured in Dulbecco's Modified Eagle Medium. Both media were supplemented with 10% foetal bovine serum and antibiotics.

C2C12 cells were transiently transfected with the pcDNA3 hTASK3/7 plasmid expressing the human TASK-3 channel protein.

## RNA interference

TASK-3 specific shRNA casettes were designed by the software of GenScript. Lipofectamine 2000 transfection reagent was used to introduce the shRNA molecules with Rodamin Red labelling on their 5' end into melanoma and HEK293 cells. The corresponding scrambled RNA casette was used as negative control. After transfection, cells were maintained in cell culture for 1, 2 or 3 more days, and then immunohistochemistry experiment were conducted on them. The most effective shRNA cassette and the corresponding scrambled sequence was also used for the creation of expression vector, by ligating the double stranded DNA encoding the cassettes into the pRNAT-U6.1/Neo plasmid. After ligation the plasmid was transformed into competent *E. coli* bacteria. After selection based on ampicillin resistance the plasmid DNA was prepared from the bacterial colonies with Wizard Plus SD Minipreps kit. The successful ligation of the insert was confirmed by gel electrophoresis after restriction enzyme digestion and DNA sequencing as well. The expression vector was also introduced into the melanoma cells with Lipofectamine 2000 transfection reagent.

## RT-PCR

Total RNA was isolated from the melanoma cells with RNeasy Micro Kit (Qiagen), and cDNS was generated by reverse transcription. Specific primer pairs were designed for the mRNA sequence of the *Homo sapiens* KCNK9 $K^+$ channel (accession number: BC075080). For the semiquantitative evaluation of RT-PCR results, glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) amplification was also carried out parallel to TASK-3. The amplified end products were separated by gel electrophoresis in 1,5% agarose gel stained with ethidium-bromid and densitometry analysis was performed on the gel image.

## Immunocytochemistry and immunohistochemistry

Immunocytochemistry experiments were carried out on cells cultured on sterile coverslips. Cells were fixed with 4% paraformaldehyde, permeabilised, and incubated overnight with the TASK-3 specific monoclonal primary antibody. After the application of fluorescein isothiocyanate (FITC) conjugateg secondary antibody cells were mounted with Vectashield medium containing DAPI. Similar immunostaining was carried out on the mitochondrial fraction isolated from melanoma cells.

Immunohistochemisry experiments were carried out on 4 μm thick formalin-fixed, paraffin-embedded tissue sections from samples that were surgically removed for postoperative histopathological diagnosis. In some cases antigen retrieval was performed before the immunoreaction. The endogen peroxidise activity was inhibited by 3% aqueous solution of $H_2O_2$. After the application of the primary antibody, the immunoreaction was visualised either by EnVision system or by biotin-conjugated secondary antibody and streptavidin conjugated with horseradish peroxidas enzyme. Negative control experiments were carried out either by preincubation with the immunising protein, or by omitting the primary antibody from the reaction.

## Western blot

SDS polyacrylamide gel electrophoresis was carried out on samples prepared from whole cell lysate after the determination of their protein content. The separated protein fractions were transferred to nitrocellulose membrane. After the blocking of aspecific protein-binding sites the membrane was incubated with a polyclonal TASK-3 specific antibody raised in rabbit, and then with a secondary antibody raised against rabbit IgG, conjugated with horseradish peroxidase. The results were visualised using an enhanced chemiluminescence Western blot detection kit. Densitometry analysis was performed for the quantitative evaluation of TASK-3 protein expression level, where the optical density was normailsed to β-actin. Samples prepared from mitochondrial membrane fraction were also used for Western-blot experiments.

## Measurement of cell proliferation and cell size

Two different methods were employed for the measurement of cell proliferation: CyQUANT assay based on the determination of the DNA content of nuclei, and MTT assay based on the measurement of mitochondrial enzyme activity proportional with the viable cell number. The size of melanoma cells was characterised by their surface area as seen under the microscope.

## Measurement of mitochondrial function

Mitochondrial function was measured on the basis of determining the extracellular concentration of reactive oxygen specied (ROS). The permanganate-based ROS determination method was developed by our team. Cells were incubated with modified HANK solution (without chloride and organic substances), and parallelly with modified HANK solution containing 1,5 mM $H_2O_2$. The ROS concentration of the supernatant was measured photometrically.

# Results

## Mitochondrial expression of TASK-3 channels in melanoma cells

It was an important finding of our earlier work that the TASK-3 expression of different cancer cells is mainly intracellular. The aim of this study was to determine which intracellular organelle is responsible for this expression pattern in melanoma cells. In our immunocytochemistry experiments conducted on cultured WM35 melanoma cells we found that the distribution of TASK-3 immunopositivity is very similar to that of mitochondrial labelling. The immunoreaction was especially strong around the nucleus, or, in the case of multinuclear tumour cells, between the nuclei as well as in the growing processes; the sites where mitochondria are also present in high amount and density. However, the immunolabelling of the cell membrane was surprisingly low. In order to demonstrate the mitochondrial localisation, TASK-3/MitoTracker® double staining was performed, and the result was examined by confocal microscopy. The thickness of optical sections was 0,5 μm throughout the whole cell layer. The images obtained this way clearly showed that the tight co-localisation does not result from the vertical overlap of the two staining pattern, but it truly indicates the presence of TASK-3 channels in the mitochondrial membrane.

The presence of TASK-3 channel protein was examined in isolated mitochondria as well. The stong co-localisation of the TASK-3 specific immunoreaction and MitoTracker® staining could also be observed in the isolated mitochondrial suspension. This result could be well reproduced with a different staining approach, where the mitochondria were labelled with a monoclonal, cytochrome-c specific antibody, and the TASK-3 channels with a polyclonal antibody.

Western blot technique was also applied to demonstate the presence of TASK-3 protein in the mitochondrial membrane fraction isolated from the WM35 melanoma cell line. In these experiments succinate dehydrogenase (SDHA), TASK-3 and $P_2X_7$ purinergic

receptor specific antibodies were employed. The TASK-3 specific band was present in the samples containing mitochondrial membrane fraction, showing that the channel protein is expressed in either the inner or the outer mitochondrial membrane. The SDHA specific band proved that the sample contained mitochondrial membrane indeed, while the lack of $P_2X_7$ specific band demonstrated that it was not contaminated with cell surface membrane fraction. Whole cell lysate of WM35 melanoma cells were applied as positive control and samples prepared from naïve (TASK-3 negative) HEK293 cells were used as negative control to demonstrate the adequate function of the TASK-3 specific antibody.

The TASK-3 expression of melanoma cells was confirmed on mRNA level as well. Total mRNA was isolated from 3 different melanoma cell lines, and it was observed that the sequence of TASK-3 specific mRNA of all three samples was completely identical with the human TASK-3 mRNA sequence available in GeneBank. This experiment not only confirmed that TASK-3 channels are expressed in melanoma cells, but it also showed that the channel protein contains no mutation.

Further control experiments were carried out to exclude the aspecific binding of the TASK-3 specific antibody used in the immunohistochemistry experiments to some other mitochondrial protein. TASK-3 negative C2C12 cells were transiently transfected with the pcDNA3 hTASK3/7 expression vector encoding the human TASK-3 protein, and then the double staining of TASK-3 channels and mitochondria was performed on the transfected cell cultures. In these experiments only a relatively small portion of cells showed TASK-3 immunopositivity, corresponding to the transfection efficiency, while the labelling of mitochondria could be seen in all cells. This result provides evidence that the applied antibody does not interact with mitochondrial proteins in an aspecific manner.

## TASK-3 immunopositivity of human keratinocytes

Our results presented above raised the question whether the mitochondrial expression of TASK-3 channels can be observed in tumour cells only, or maybe other, non-malignant cell types also show this expression pattern. As, according to our preliminary experiments, HaCaT human keratinocyte cells are intensely TASK-3 positive, we decided to use this cell line as the model of non-malingant cells in this work. HaCaT keratinocyte cultures showed strong TASK-3 immunopositivity with a distribution pattern similar to that of melanoma cells; there was a strong co-localisation with the mitochondrion specific labelling, which indicated that TASK-3 channels are present in the mitochondria of HaCaT cells as well.

The TASK-3 expression of keratinocytes was examined *in situ*, in vax-embedded tissue sections from healthy skin samples as well. Several cell types of the healty skin were unambiguously and strongly TASK-3 positive. Besides the keratinocytes of the squamous epithelium, the secretory cells and keratinocytes of the hair follicles also produced TASK-3 immunopositivity, while the surrounding connective tissue remained negative. The TASK-3 specific labelling of the cytoplasm often showed a granular pattern, which was most pronounced around the nuclei. This characteristic distribution pattern seems to confirm the mitochondrial TASK-3 expression of this cell type as well.

## RNA interference

Although our immunocytochemistry and Western blot experimenst convincingly showed the presence of TASK-3 channels in the mitochondrial membrane of melanoma cells, these experiments were not suitable for examining the functional significance of the channel protein. RNA interference technique was applied for this purpose, in order to examine the changes in appearance and behaviour of TASK-3 knockdown melanoma cells.

Before the interpretation of the experimental results it was absolutely important to confirm the effectivity and specificity of the applied shRNA sequence, therefore several different methods were applied to demonstrate the reduction in TASK-3 expression level. Transfection of melanoma cells with the shRNA cassettes markedly decreased TASK-3 expression on both mRNA and protein level. The reduction of both mRNA and protein expression level was statistically significant on the 2nd day after transfection, and it remained significant until the 4th day. After this period, however, the non-transfected cells tended to overgrow the ones whose TASK-3 biosynthesis was interfered with, so no further experiments were performed from the 5th day on.

The efficiency of transfection with the shRNA expression vector was examined by immunocytochemistry as well. The TASK-3 immunoreaction of knockdown melanoma cells was much less intensive than that of the cells transfected with the scrambled RNA expression vector. Besides the TASK-3 specific staining, immunoreaction with an S100 (a calcium-binding protein expressed by melanoma cells) specific antibody was also performed. There was no difference in the intensity of S100 immunoreaction between the two cell cultures.

## Morphological changes in TASK-3 knockdown melanoma cells

After the application of the shRNA cassette, characteristic morphological changes could be noted in the melanoma cells. The 2nd day after transfection differential interference contrast images were obtained from the control and TASK-3 knockdown melanoma cell cultures. The most prominent change in cell morphology was the rough granulation in the cytoplasm of shRNA-treated cells.

Images obtained from the TASK-3 specific immunostaining of shRNA-transfected melanoma cells also provided interesting information about the altered cell morphology. The successfully transfected cells became smaller compared to the control, lost their processes, and showed much weaker TASK-3 positivity than the surrounding, healthy-

looking cells. The size of successfully transfected and apparently uneffected cells was quantitatively analysed by determining their cell surface area. A marked reduction in cell size could be demonstrated in the transfected cells. The strong granulation of cytoplasm was visible on the immunocytochemistry images as well. Some of these granules showed weak, but clear TASK-3 immunopositivity. The distribution, morphology and size of these granules raised the possibility that they might have corresponded to swollen mitochondria. In the shRNA-transfected cells the nuclear TASK-3 positivity was substancially reduced or entirely absent, and when they were labelled with DAPI the nuclear material of TASK-3 negative nuclei was much more condensed than those of the non-transfected cells. The morphological traits described above could be seen in the $5.6 \pm 2.2\%$ of scrambled RNA-treated cells, while in the cultures transfected with the specific shRNA this value was $64.3 \pm 21.6\%$.

## Decrease of DNA-content and mitochondrial function

The inhibition of TASK-3 channel biosynthesis did not only affect the size and morphology of cells, but it also decreased the DNA content and mitochondrial activity of cells. The reduced amount of DNA presumabely indicated a reduced rate of cell division, while the result of MTT assay could be the consequence of decreased cell number, or the reduced mitochondrial activity of transfected cells. Since the mitochondrial localisation of TASK-3 channels along with the morphological changes in the transfected melanoma cells suggested compromised mitochondrial function, a quantitative assessment was performed by measuring the resting and ROS-induced ROS production. Although there was no significant difference between the resting ROS production of the control and transfected cells, when ROS production was induced by extracellularly applied $H_2O_2$, the knockdown cells gave a significantly lower response. These observations indicated that the lack of TASK-3 channels compromised mitochondrial function, and impaired the ability of cells to adequately react to metabolic challenges.

## Discussion

Apart from their many physiological functions, TASK-3 channels also play important role in certain non-physiological processes. The most contradictory characteristics of their function is that under certain circumstances they promote apoptotic cell death, while in other cases their activity mediates anti-apoptotic, and thus tumourigenic effect. Expression of TASK-3 channels has been reported in breast, colorectal and gastric cancers, and the overexpression of the channel protein has been described in a lung carcinoma cell line (Ben cell line).

In an earlier study we have already reported the intensive TASK-3 immunopositivity of cultured melanoma malignum cells. However, in the present work we noted that the distribution of TASK-3 channels in melanoma cells was rather surprising: the intracellular labelling was especially strong, with a characteristic granular pattern, while the cell membrane showed no significant immunopositivity. The experimental results presented in this study provide explanation for this interesting phenomenon by demonstrating that TASK-3 channels are expressed in the mitochondria.

The mitochondrial localisation of TASK-3 channels has already been described earlier by others under both *in vivo* and *in vitro* circumstances. It is known that the adapter protein called 14-3-3 is responsible for the trafficking of the channel protein into the cell surface membrane. This adapter protein binds to the C-terminus of the channel protein, and its presence is indispensable in the trafficking of TASK-3 channels into the cell membrane, hovewer the exact role of the adapter protein in this process is still unknown. In *Xenopus* oocytes the overexpression of the 14-3-3 adapter protein increased the TASK-3 current amplitude through the cell membrane, while the modification of the C-terminus of the channel protein, which inhibits the binding of the adapter protein, resulted in the significant decrease of TASK-3 current amplitude. The deletion of a single amino-acid from the

C-terminus of the protein was enough to completely abolish the binding of the adapter protein, and inhibit the TASK-3 expression in the cell surface membrane. Hovewer, in our case the mutation of the channel protein could be excluded, as no mutation could be observed in the sequence of mRNA isolated from melanoma cells. An alternative explanation can be that the adapter protein itself is missing or modified in melanoma cells.

The intracellular presence of TASK-3 channels has also been described in the central nervous system of the rat, where the Golgi apparatus, the endoplasmic reticulum and some intracellular vesicles showed the most prominent labelling, hovewer the mitochondria and nuclei were TASK-3 negative. Based on these earlier findings we assumed that the intracellular presence of TASK-3 channel in melanoma cells is also explained by the expression of the channel protein in some intracellular organelles. In our experiments double labelling of TASK-3 channels and mitochondria revealed strong co-localisation, which indicated the mitochondrial presence of TASK-3 channels. This observation raised the question whether the mitochondrial expression of TASK-3 channels is functionally important, and if yes, what is their role, and where exactly are they expressed in the mitochondria.

The results of our present work confirmed that TASK-3 channels play an important role in maintaining mitochondrial function. The inhibition of the channel protein's synthesis caused characterisctic morphological changes in the cell cultures, the decrease of cell size, DNA content and the metabolic activity. These changes indicate that functional TASK-3 channels might be crucially important in the survival and proliferation of melanoma cells.

Although the results of this study do not provide information about the exact localisation and function of TASK-3 channels within the mitochondria, some possibilities can be mentioned. Some $K^+$-permeable channels are known to be present in the inner mitochondrial membrane: for example, the ATP-sensitive $K^+$-permeable channels (mtKATP) are involved in the regulation of mithochondrial volume as well as in maintaining the proton

and transmembrane electrochemical gradient which is indispensable in the mitochondrial oxidative processes. TASK-3 channels, if they are expressed in the inner membrane, might have similar function. However, it cannot it cannot be excluded that they are localised in the outer mitochondrial membrane, in which case they might have a similar role to that of Bcl channels. Functional Bcl channels serve as cation-permeable pores; the mitochondrial membrane is hyperpolarised upon their activation, which inhibits the release of cytochrome-c and thus helps maintaining the mithochondrial osmotic homeostasis.

It is a very interesting phenomenon that TASK-3 channels, under certain circumstances show anti-apoptotic, tumourigenic finction, while in other cases (such as in cerebellar granule neurones) they have pro-apoptotic function, which is necessary for the physiological apoptosis of migrating granule cells. Although the exact reason of this contradictory behaviour in unknown, there are several theories for the explanation of this phenomenon. It was proposed for example, that some key elements of the apoptotic pathway might be missing from tumour cells. Hovewer in the case of the melanoma cells investigated in this work this is not an option, as the application of 2-methoxi-estradiol could induce apoptosis in these cell cultures.

The contradictory behaviour might also be in connection with the amplitude of TASK-3 current across the cell membrane, which is 1-2 nA in the case of cerebellar granule cells, while it is only 100 pA in the malignant Ben cells, even though this cell line actually overexpresses the channel protein. It is possible that TASK-3 channels are expressed intracellularly in Ben cells, and perhaps contribute to cell survival by supporting their mitochondrial function. This hypothesis also explains why the TASK-3 current amplitude in the cell surface membrane does not reflect the fact that TASK-3 channels are overexpressed in this cell line.

If any of the above mentioned possibilities is correct, it can be assumed that the localisation of TASK-3 channels determines whether they mediate cell death or increased tolerance against hypoxia and serum deprivation. The activation of TASK-3 channels in the cell surface membrane leads to $K^+$-efflux, which in turn causes apoptotic volume decrease, and cell death. TASK-3 channels situated in the mitochondria (e.g. in melanoma cells), however might have a positive effect on mitochondrial function, and thus enhance the process of ATP production.

The role of TASK-3 channels in ensuring the survival of malignant melanoma cells suggests that they might be taken into consideration as possible therapeutic targets. However it also must be pointed out that TASK-3 channels are expressed in several non-malignant cell types as well, such as cerebellar Purkinje and granule cells, endocrine cells in the Langerhans islets of pancreas and healthy melanocytes and keratinocytes. TASK-3 channels are also present in the intestinal epithelium and neuronal elements of the gastrointestinal trackt, whereas the connective tissue and the smooth muscle cells did not show TASK-3 positivity. This implies that the inhibition of TASK-3 channel function could be a possible therapeutic approach only if it was carried out in a target cell specific manner.

Taken together our experimental data suggest, that TASK-3 channels are functionally present in the mitochondria of malignant melanoma cells. This observation does not only elucidate the background of the intracellular TASK-3 expression, but it also might provide the previously missing link between TASK-3 channel function and increased survival of tumour cells.

## Summary

TASK-3 channels are expressed in several cell types including both healthy and malignantly transformed cells. The role of channel function in tumourigenesis has been proposed in different human cancers. In this work the distribution pattern of TASK-3 channel protein was investigated in cultured melanoma malignum cells and in non-malignant HaCaT keratinocytes using immunochemical methods. Before these experiments, the TASK-3 expression of melanoma cells was confirmed on mRNA level as well. The subcellular localization of the channel protein was found to be mainly intracellular, while the labelling of cell surface membrane was not significant. Double-labelling experiments revealed that the distribution of TASK-3 channels shows strong co-localisation with mitochondria in both investigated cell types. These results were confirmed by immunohistochemical staining of wax-embedded human skin tissue sections, where the same labelling pattern could be observed in melanocytes and keratinocytes as well.

Our findings suggested that TASK-3 channels are present in the mitochondrial membrane of melanoma cells, where they might contribute to mitochondrial function. It has been proposed earlier that they might provide greater hypoxia tolerance for tumour cells, which might contribute to cell survival and tumour growth, but the exact mechanism by which these effects are exerted is still unknown.

We investigated the significance of TASK-3 mitochondrial TASK-3 channels in cell viability by interfering with the function of the channel protein. Melanoma cells were transiently transfected with shRNA cassettes, and TASK-3 knockdown cell cultures were generated. The reduced expression of TASK-3 resulted in characteristic changes of the cell morphology, reduction of DNA content, decreased metabolic activity and impaired mitochondrial function. These changes indicate that TASK-3 channels expressed in the mitochondrial membrane may have crucial roles in maintaining the viability of melanoma

cells. However, further experiments are needed to clarify whether the inhibition of TASK-3 channel function could be exploited in anti-cancer therapy.

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**List of publications related to the dissertation**

1. Rusznák, Z., Bakondi, G., **Kosztka, L.**, Pocsai, K., Dienes, B., Fodor, J., Telek, A., Gönczi, M., Szűcs, G., Csernoch, L.: Mitochondrial expression of the two-pore domain TASK-3 channels in malignantly transformed and non-malignant human cells.
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2. **Kosztka, L.**, Rusznák, Z., Nagy, D., Nagy, Z., Fodor, J., Szűcs, G., Telek, A., Gönczi, M., Ruzsnavszky, O., Szentandrássy, N., Csernoch, L.: Inhibition of TASK-3 (KCNK9) channel biosynthesis changes cell morphology and decreases both DNA content and mitochondrial function of melanoma cells maintained in cell culture.
*Melanoma Res. 21* (4), 308-322, 2011.
DOI: http://dx.doi.org/10.1097/CMR.0b013e3283462713
IF:2.254 (2010)

**List of other publications**

3. Pocsai, K., **Kosztka, L.**, Bakondi, G., Gönczi, M., Fodor, J., Dienes, B., Szentesi, P., Kovács, I., Feniger-Barish, R., Kopf, E., Zharhary, D., Szűcs, G., Csernoch, L., Rusznák, Z.: Melanoma cells exhibit strong intracellular TASK-3-specific immunopositivity in both tissue sections and cell culture.
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*Melanoma Res. 21* (1), 2010.
DOI: http://dx.doi.org/10.1097/CMR.0b013e3283414477
IF:2.254

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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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