DIPLOMARBEIT

Titel der Diplomarbeit

Establishing an *in vitro* system for analysis of melatonin receptor MT1 function in trophoblast cells: relevance for preeclampsia

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THIS DIPLOMATHESIS IS DEDICATED TO MY PARENTS
AND MY BROTHER ALEXANDER

Acknowledgments

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1 Abstract

1.1 Abstract

Melatonin, also known as the hormone of the darkness, is a lipophilic indolamine produced and secreted mainly during the night from the pineal gland. Immediately after secretion, it enters the circulation and exerts a plethora of functions on diverse cells and tissues before degradation occurs. Some of the major functions of melatonin in humans are mediated via the high-affinity, G-protein-coupled melatonin receptors MT1 and MT2. Via these receptors, melatonin can promote sleep, participates in blood pressure regulation, exerts immunomodulatory and oncostatic effects or functions as a chronobiotic. In humans, maternal melatonin can also cross the placenta and thereby transmits photoperiodic information to the fetus. In addition, there is increasing evidence that melatonin can regulate placental cell growth and function via melatonin receptors and therefore seems to play a role in the occurrence of preeclampsia. These effects, however, require systematic investigations.

This study aimed to establish an *in vitro* system for placental trophoblast cells exhibiting endogenous and induced expression of human MT1 to facilitate future characterization of MT1 function in human placental trophoblast cells. A second aim of the study was the characterization of three anti-MT1 antibodies by western blotting.

Due to its trophoblast-like features, the choriocarcinoma cell line BeWo was selected as *in vitro* system, and the endogenous expression of MT1 mRNA was confirmed by two different RT-PCR protocols. A lipid-based transfection protocol was established and the cells were transfected with three different plasmids containing human MT1 cDNA linked to various tags (flag, HA, GFP) that would enable anti-MT1 independent detection at the protein level. Within 24 to 48 hours after transfection, upregulation of MT1 mRNA in flag- and HA-tagged MT1 cDNA transfected BeWo cells was confirmed. Following transient transfection, cells were subjected to antibiotic treatment to perform selection of stable transformed cells. Although geneticin-resistant cell clones were obtained, in none of these clones expression of either a flag- or a HA-tagged protein was demonstrated. In addition, three different anti-MT1 antibodies were tested by western blotting for their ability to detect endogenous MT1 protein on BeWo cell lysates. One antibody reacted with several proteins in the lysate, while two antibodies showed a more specific binding pattern. One of these antibodies recognized only a 40 kDa protein that might represent the non-glycosylated form of MT1, while the other antibody detected mainly a 60 kDa protein that could represent the glycosylated form of MT1. Especially the latter antibody could prove to be an important tool in future investigations of MT1 functions in trophoblast cells.

Transient increase of MT1 mRNA has been demonstrated in BeWo cells following transfection with flag- and HA-tagged MT1 cDNA, unfortunately, this increase in mRNA was not accompanied by any detectable increase in MT1 protein expression in stable transformed clones. Since many studies involved in characterization of MT1 protein function have been performed following transient transfection of the MT1 constructs into non-polarized cell lines, it remains to be analyzed whether high levels of flag-tagged or HA-tagged protein can be observed in BeWo cells only immediately after (transient) transfection or whether high expression of MT1 protein in BeWo cells is always limited by yet unknown MT1-regulating endogenous factors.

In order to generate an *in vitro* system to study MT1 functions in human placental trophoblast cells, the choriocarcinoma cell line BeWo has been demonstrated to express MT1 mRNA endogenously and – following transient transfection with MT1 cDNA – MT1 mRNA levels were increased. This *in-vitro* system will serve as a tool for (1) future analysis of MT1 function in human placental trophoblast cells and (2) further characterization of two anti-MT1 antibodies used in this study. This will provide an important *in vitro* model to investigate mechanisms of preeclampsia and a possible involvement of melatonin to this disease.

1.2 Abstrakt

Melatonin ist ein lipophiles Indolamin, das von der Zirbeldrüse produziert und sezerniert wird. Da dieses vor allem nachts produziert und freigesetzt wird, nennt man es auch das "Hormon der Finsternis". Sofort nach der Sekretion wird es in die Zirkulation entlassen und leitet verschiedenste Prozesse in unterschiedlichen Zellen und Geweben ein, bevor es wieder degradiert wird. Einige seiner wichtigsten Funktionen mediiert Melatonin über zwei hoch-affine, G-Protein gekoppelte Melatonin-Rezeptoren, MT1 und MT2. Über MT1 und MT2 fördert Melatonin den Schlaf, nimmt an der Blutdruckregulation teil, zeigt immunmodulatorische und onkostatische sowie chronobiotische Effekte. Beim Menschen durchquert mütterliches Melatonin auch die Plazenta und übermittelt dem Föten so wichtige fotoperiodische Informationen. Rezente Untersuchungen zeigen, dass Melatonin über Rezeptoren auch das Wachstum plazentarer Zellen beeinflussen kann, und dass MT1 und MT2 in der Plazenta exprimiert sind. Dies indiziert die Annahme, dass Melatonin auch eine Rolle in der Entstehung der Preeklampsie spielen könnte. Diese Effekte bedürfen allerdings einer systematischen Untersuchung.

In dieser Studie sollte ein *in vitro* System für plazentare Trophoblasten etabliert werden, in dem einerseits endogene Expression von MT1 gezeigt, andererseits die Expression von MT1 auch induziert werden sollte, um zukünftige Untersuchungen der Funktionen von

MT1 in plazentaren Trophoblasten einfacher durchführen zu können. Ein weiteres Ziel der Studie war die Charakterisierung von drei verschiedenen anti-MT1 Antikörpern mittels Immunoblotting.

Die von Trophoblasten abstammende Choriokarzinomzelllinie BeWo wurde als *in vitro* System gewählt. Die endogene Expression von MT1 mRNA wurde mittels zweier unterschiedlicher RT-PCR Protokolle bestätigt. Ein Lipid-basierendes Transfektionsprotokoll wurde etabliert. Anschließend wurden die BeWo Zellen mit drei unterschiedlichen Plasmiden transfektiert. Die drei Plasmide kodieren für humane MT1 cDNA verknüpft mit unterschiedlichen "Markierungen" wie flag, HA oder GFP, die eine spätere anti-MT Antikörper-unabhängige Detektion auf Proteinebene ermöglichen sollten. 24 bis 48 Stunden nach der Transfektion mit flag- und HA-markierter cDNA von MT1, wurde die erhöhte Expression von MT1 mRNA in den transfektierten BeWo Zellen bestätigt. Nach dieser transienten Transfektion wurden die Zellen in Gegenwart von Antibiotikum weiterkultiviert, um stabil transformierte Zellen zu selektieren. Obwohl Geneticin-resistente Zellklone generiert wurden, konnte in keinem dieser Klone eine Expression von entweder flag-markiertem oder HA-markiertem Protein festgestellt werden. In dieser Studie wurden weiters drei unterschiedliche anti-MT1 Antikörper mittels Immunoblot hinsichtlich ihrer Fähigkeit getestet, endogenes MT1-Protein in BeWo Zelllysaten zu detektieren. Einer der Antikörper reagierte mit mehreren Proteinen in den Zelllysaten, während die anderen beiden Antikörper ein spezifisches Reaktionsmuster erkennen liesen. Einer dieser beiden Antikörper erkannte nur ein ca. 40 kDa Protein, das die nicht-glykosilierte Form von MT1 Protein darstellen könnte, während der andere der beiden Antikörper hauptsächlich mit einem ca. 60 kDa Protein reagierte, das der glykosilierten Form des Rezeptors entsprechen könnte. Besonders dieser letzte Antikörper scheint ein vielversprechendes Werkzeug für zukünftige Untersuchungen von MT1 in Trophoblasten darzustellen.

Eine transiente Zunahme von MT1 mRNA wurde in BeWo Zellen nach Transfektion mit flag- und HA-markierter MT1 cDNA festgestellt, leider wurde dieser mRNA Anstieg aber nicht von einer Zunahme der Proteinexpression in den stabil transformierten Zellklonen begleitet. Da viele Studien, die sich mit der Charakterisierung von MT1-Funktionen beschäftigten, unmittelbar nach transienter Transfektion von MT1 Konstrukten in nicht-polarisierten Zelllinien durchgeführt wurden, muss noch ermittelt werden, ob eine Zunahme an flag-markierten or HA-markierten MT1 Proteinexpression in BeWo Zellen nur unmittelbar nach einer (transienten) Expression erfolgt oder ob eine hohe Expression von MT1 Protein in BeWo Zellen immer durch bis jetzt unbekannte Faktoren limitiert sein könnte.

Um ein *in vitro* System zu schaffen, in dem die Funktionen von MT1 in humanen, plazentaren Trophoblasten untersucht werden können, wurde in dieser Arbeit gezeigt, dass die Choriocarzinomzelllinie BeWo MT1 mRNA endogen expremiert und nach erfolgter Transfektion von MT1 cDNA die MT1 mRNA Werte erhöht wurden. Dieses *in-vitro* System wird in Zukunft als Werkzeug für einerseits die Analyse der MT1 Funktionen in humanen plazentaren Trophoblasten dienen und andererseits mithelfen die bereits in dieser Studie untersuchten anti-MT1 Antikörper weiter zu charakterisieren. Das etablierte Modell dient als wichtige Grundlage für genauere Untersuchung der Präeklampsie. Weiters könnte die Rolle des Melatonins in dieser Erkrankung näher untersucht werden.

2 Introduction

Melatonin (N-acetyl-5 methoxytryptamine), also known as the hormone of the darkness, was first discovered by Aaron Lerner and coworkers in 1958 in extracts of bovine pineal gland ([1]). In later years, the functional activity of melatonin was demonstrated in many organisms ranging from bacteria to mammalians, including humans ([2]; [3]) [4]. In vertebrates, synthesis and release of melatonin was originally described to occur in the pinealocytes of the pineal gland (see figure 1; [5]). The biosynthesis of pineal melatonin is strictly regulated by the circadian clock of the suprachiasmatic nuclei (SCN) of the hypothalamus. Therefore, the secretion of the indolamine follows a circadian rhythm that is conserved across vertebrate species ([6]). Pineal melatonin production shuts down when light signals are transmitted from the retina to the pineal gland, but increases during the dark. In humans, peak secretion occurs in the middle of the night, between 2 and 4 a.m. ([7]). During the periods of increased synthesis, melatonin is released into the cerebrospinal fluid and reaches the circulation. As this indolamine displays high lipid solubility, it can easily cross all cellular membranes ([8]).

Melatonin is a systemically acting chronobiotic that exerts multiple actions, either directly or indirectly, before it is rapidly metabolised. In many species, it plays an important role in the transmission of photoperiod information to physiologic processes that vary with seasons, such as reproduction, pelage, sleep, body weight and appetite. It is also associated with circadian rhythms, influencing sleep propensity and the core temperature rhythm ([9]). Involvement of melatonin in other physiological and pathological processes like aging and age-related diseases, immunomodulation, blood pressure regulation, gastroprotection or oncogenesis is supported by a multiplicity of studies ([10]; [4]; [11] [7]; [12]; [13]; [14]; [15]; [16]; [17]; [18]).

In addition to the long known regulation of seasonal reproductive events in photoperiod-dependent long-day and short-day breeding mammals ([19]), more recent data demonstrating activity of melatonin-synthesizing enzymes and expression of receptors for melatonin in the human placenta ([20]; [21]; [22]) suggest that human placental function and consequently pregnancy are also affected by melatonin (see chapter 2.5).
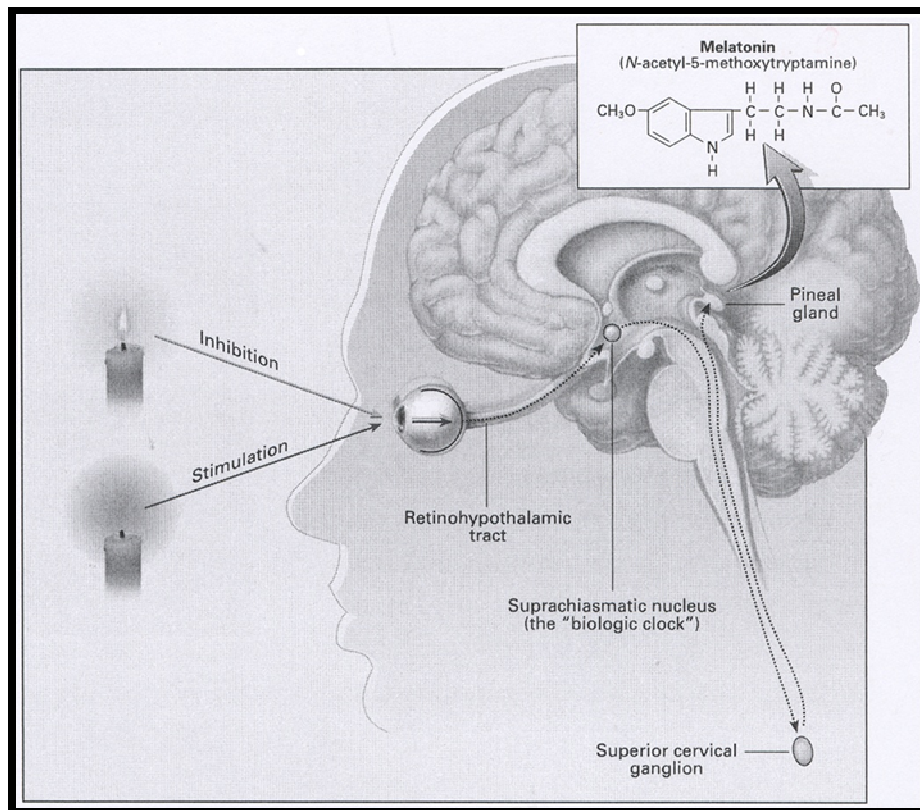
Figure 1: Melatonin is a lipophilic indolamine which is to a significant part produced and released by the pineal gland. Its synthesis and secretion follows a circadian rhythm with peak secretion occuring the night **(**Source: [7])

2.1 Biosynthesis of melatonin

The classical way for the pineal synthesis of melatonin starts with the uptake of the amino acid tryptophan from serum into the pineal gland (see Figure 2). Tryptophan is converted to serotonin via 5-hydroxytryptophan. Serotonin is then acetylated via arylalkylamine N-acetyltransferase (AA-NAT). The resulting N-acetylserotonin is finally metabolised to melatonin by HIOMT (Hydroxyindole-O-methyltransferase; [23]; [6]).
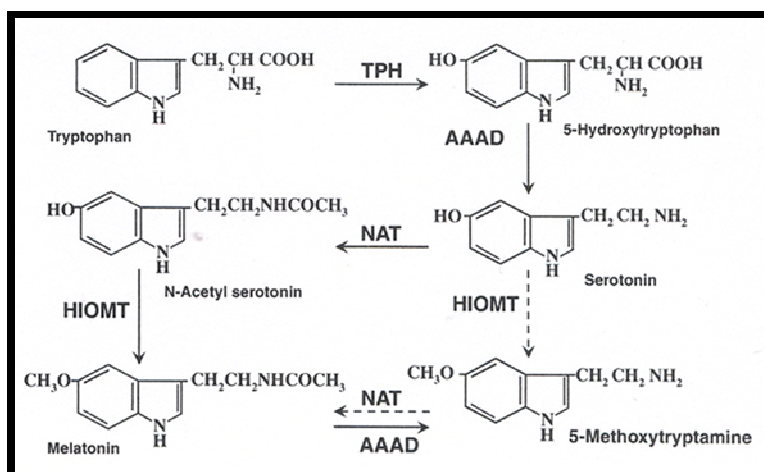
Figure 2: The synthesis of melatonin starts from the amino acid tryptophan. Tryptophan is then converted into serotonin acetylated via (AA-)NAT (arylalkylamine N-acetyltransferase) and finally metabolized to melatonin via HIOMT (Hydroxyindole-O-methyltransferase; Source: ([12])

In all species, AA-NAT represents a key enzyme in the synthesis of melatonin, which is responsible for the diurnal rhythms of secretion. For a long time, this enzyme was suggested to be the overall rate-limiting enzyme of melatonin production. More recent data, however, demonstrate that the *in vivo* rate of formation of melatonin during the night is determined by HIOMT activity ([23]). The activity of the enzyme AA-NAT can be stimulated via different mechanisms, namely by cAMP-dependent transcriptional activation or proteinkinase A (PKA)-mediated post-transcriptional control determining enzyme activation and stability. The applied mechanism depends on the species and organ under investigation ([23]; [11]). Overall, the pineal melatonin synthesis is under the influence of the retinohypothalamic tract that transmits information about light or darkness from the retina to the SCN, which is the major circadian oscillator. This information is then passed to the pineal gland by nerve cells that in the dark release norepinephrine (NE). The binding of NE to either ß-adrenergic or α_{1B}-adrenergic receptors on pinealocytes activates cAMP levels, leads to phosphorylation of CREB (Ca^{2+}/cAMP response element binding proteins) and subsequently to the synthesis of melatonin synthesizing enzymes. The melatonin syntesis is potentiated by vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide and neuropeptide Y ([4];[11]).

Although, melatonin was first identified in pinealocytes, and the pineal gland is responsible for the light/dark cycle of melatonin, this organ is not the only source of melatonin. The gastrointestinal tract, for example, produces melatonin in serotonin-rich entero-endocrine cells in about 500 times larger amounts than the pineal gland. Melatonin can exert its function in the gastrointestinal tract in a paracrine way or it is released as a hormone into

the blood stream, thereby being responsible for a large fraction of the day time serum melatonin concentrations ([14]). Other studies demonstrated melatonin production with a circadian rhythm in the retina, and suggested melatonin to participate in many of the ocular functions ([24]). To date, extrapineal melatonin synthesis has been identified in the brain, Harderian gland, ciliary body, lens, thymus, airway epithelium, bone marrow, immune cells, and skin ([4]; [25]; [20]). Additionally, AA-NAT and HIOMT activities as indicators for melatonin production, have been demonstrated in the human placenta ([22]; [4]).

2.2 Metabolism of melatonin

Following its release from the pineal gland (or other tissues) into the circulation, more than 90% of the circulating lipophilic hormone melatonin is metabolised by the liver. Melatonin is first hydroxylated by a hepatic cytochrome P450, mainly CYP1A2. The product, 6-hydroxymelatonin is then conjugated either with sulphate or with glucuronic acid and the conjugates are eliminated via urine. This metabolism is rapid, ranging between 10 and 60 minutes in humans and therefore, the urinary excretion of 6-hydroxymelatonin closely reflects the plasma melatonin profile. It is consequently often used to evaluate human melatonin rhythms.

The metabolism in extrahepatic tissues differs substantially. In the retina, but also pineal gland (i.e. tissues of neural origin), melatonin is deacetylated by aryl acylamidases to 5-methoxytryptamine that is further processed by deamination followed by oxidation or reduction. In the brain, melatonin is metabolised differently. Oxidative pyrrole-ring cleavage produces AFMK (N^{1}-acetyl-N^{2}-formyl-5-methoxykynuramine) that is further metabolised to AMK (N^{1}-acetyl-5-methoxykynuramine). AFMK and AMK also result from the action of melatonin as a free radical scavenger with reactive oxygene species (ROS) and reactive nitrogen species (RNS). These metabolites are of great interest as they share the anti-oxidant and anti-inflammatory properties of melatonin.

Apart from the metabolism of melatonin via enzymes, non-enzymatic metabolism occurs in all cells or even outside the cells due to interaction with free radicals and other oxidants (see chapter 2.3.1). If melatonin scavenges two hydroxyl radicals, cyclic 3-hydroxymelatonin is formed. This product is regarded as a biomarker for endogenous hydroxyl radical levels ([9]; [12]; [4]; [11]).

2.3 Mechanisms for melatonin action

Melatonin can exert its action by different mechanisms (see Figure 3) that in principle are either receptor-independent (direct radical scavenging activity of melatonin) or require melatonin receptors (part of its anti-oxidant activity and all other functions) ([4])
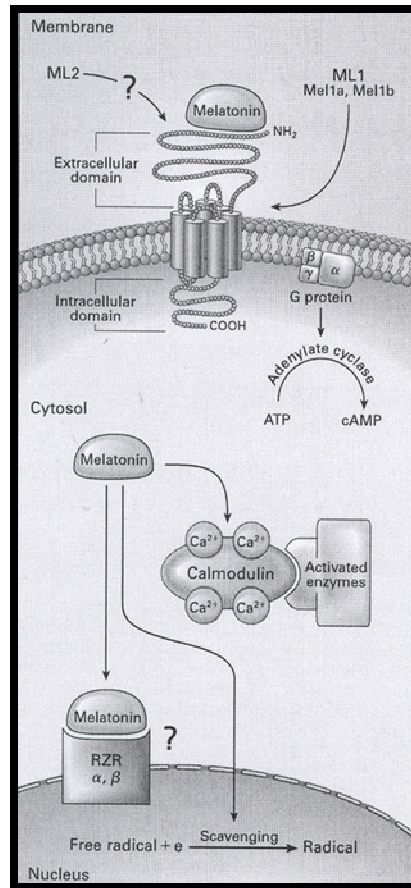
Figure 3: The effects of melatonin are mediated either in a direct or an indirect way. The hormone can act indirectly via receptors located in the membrane (MT1 and MT2) or in the nucleus (RZR) as well as cytoplasmic proteins (calmodulin) or directly as free radical scavenger (Source: [7])

2.3.1 Anti-oxidant actions of melatonin

Free radicals can destroy cells and may participate in development of diseases like cancer, neurodegeneration and autoimmune conditions. Anti-oxidants, like melatonin, eliminate these free radicals from the cells. In a situation of oxidative stress, the balance between free radicals and anti-oxidative systems of the cell is distroyed ([26]).

As an anti-oxidant, one molecule of melatonin can directly scavenge two hydroxyl radicals ($OH^{.}$) resulting in the formation of cyclic 3-hydroxymelatonin (3-OHM), that by itself is a free radical scavenger ([27]; [28]). The indolamine also protects from other ROS and RNS

([27]). While the RNS NO$^{\cdot}$ by itself is not very dangerous, it interacts with O_2^{-} to form the peroxynitrite anion (ONOO^{-}; [27]), which attacks cellular macromolecules like lipids, proteins and DNA. Formation of ONOO^{-} may be avoided by melatonin ([29]; [30]) or, once formed, ONOO^{-} may be detoxified by melatonin to 1-nitrosomelatonin and 1-hydroxymelatonin through a nitration and a hydroxylation of the pyrrole nitrogen ([31]; [32]). Although it is not completely clear yet, whether melatonin can by itself scavenge the radical LOO$^{\cdot}$ arising from the peroxidation of lipids ([33]; [34]; [35]), melatonin might reduce the peroxidation of lipids by scavenging its initiating agents ([27]).

Melatonin, however, has not only a direct antioxidative activity, but it can also stimulate enzymes involved in oxidative stress defense via different melatonin receptors (see below 2.3.2) ([27]; [4]).

For example, the molecule H_2O_2 is not very reactive itself, but can be converted to the more reactive OH$^{\cdot}$. This is usually prevented by elimination of H_2O_2 from the cell through the action of the enzyme glutathione peroxidase GPx, (also named GSH-Px) accompanied by oxidation of glutathione (GSH). Melatonin can increase either the activity of GPx as demonstrated in human chorion ([36]) or it can upregulate the enzyme glutathione reductase (GRd) that reduces oxidized GSH ([27]). GRd requires the co-factor Nicotinamide adenine dinucleotide phosphate (NADPH); NADPH is formed by the enzyme glucose-6-phosphate dehydrogenase (G6PD) that is also stimulated by melatonin ([37]; [27]). Finally, GSH synthesis is mediated by the rate limiting enzyme γ-glutamylcysteine synthetase (γ-GCS) that can be upregulated by melatonin following binding to the melatonin receptor RZR/RORα ([26]; [27]; [38]). Conversion of H_2O_2 can also be accomplished by the enzyme catalase (CAT), which separates two H_2O_2 molecules into two molecules of water and one diatomic oxygen. CAT is up-regulated by melatonin, too ([26]; [39]; [27]). As another example, the enzyme superoxide dismutase (SOD) is augmented by melatonin in the fetal rat brain ([40]).

Additionally, melatonin inhibits enzymes producing free radicals, like the lipoxygenase ([41]) and nitric oxide synthase ([42]; [43]).

2.3.2 Interaction of melatonin with receptors or binding proteins

Some major melatonin-mediated actions require binding to the plasma membrane receptors, MT1 and MT2, formerly termed Mel 1a or Mel 1b. As an example, these receptors mediate the chronobiological effects at the SCN, by either suppressing the neuronal firing activity (MT1), or acting mainly by inducing phase shifts (MT2). In the peripheral tissue and cells, they are, for example, involved in vasomotor control or

immunomodulatory functions ([44]). Characteristics of these receptors are described below in more detail.

Other melatonin binding sites are less well described, but some of them may be of particular importance for the actions of melatonin produced in extra-pineal tissues.

Quinone reductase 2

Melatonin binds to the enzyme quinone reductase 2 (QR 2) that is found in tissues like kidney, brain, liver and testes ([45]). Originally thought to be another membrane receptor and therefore named MT3, it turned out to be an enzyme mostly localized in the cytosol. The affinity of the indolamine for this enzyme is lower than for MT1 and MT2, resulting in a fast association and dissociation. The role of QR 2 is to protect against oxidative stress, preventing the electron transfer reactions of quinones. Despite the existence of several hypotheses, the action of melatonin on this enzyme is not really understood ([46]; [11]; [47]; [4]).

Nuclear binding sites

Nuclear binding sites of melatonin include melatonin-binding transcription factors belonging to the retinoic acid receptor superfamily. The complex of ligand/receptor binds to special sequences of the DNA, known as ROREs, which are hormone response elements ([48]) and can influence gene transcription ([49]). The family encloses three receptors: RORα, including its four splicing variants RORα1, RORα2, RORα3 and RZRα, RZRβ and RORγ. The affinity of melatonin to the nuclear receptors is not very high, with K_d values in the lower nanomolar range ([11]; [4]; [50]; [49]). Many aspects of their melatonin-associated function await characterization, since most of the studies so far only demonstrated ROR/RZR expression. RORα1 and RORα2 seem to be involved in some aspects of immune modulation ([4]), while RORα might mediate upregulation of antioxidant enzymes ([51]) or upregulation of HIF-1α (hypoxia-inducible factor 1α; [52]) .

Interaction of melatonin with intracellular proteins

Melatonin can bind to calmodulin or to calreticulin, but the likeliness and consequences of these interactions remain to be characterized ([11]). Additional binding sites may exist, for instance in the mitochondria, but they also await their characterization ([53] ; [54]).

Melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2)

MT1 and MT2 belong to the superfamily of G protein-coupled receptors (GPCRs) that exhibit high (in the picomolar range), though slightly different affinity to (^{125}I-)melatonin ([55]). In mammals, only MT1 and MT2 are expressed ([56]; [57]), while in non-

mammalian vertebrates a third receptor type, Mel1c, exists ([58]). Both MT1 and MT2 have seven transmembrane α-helices that are linked by three alternating intracellular and extracellular loops (see Figure 4).
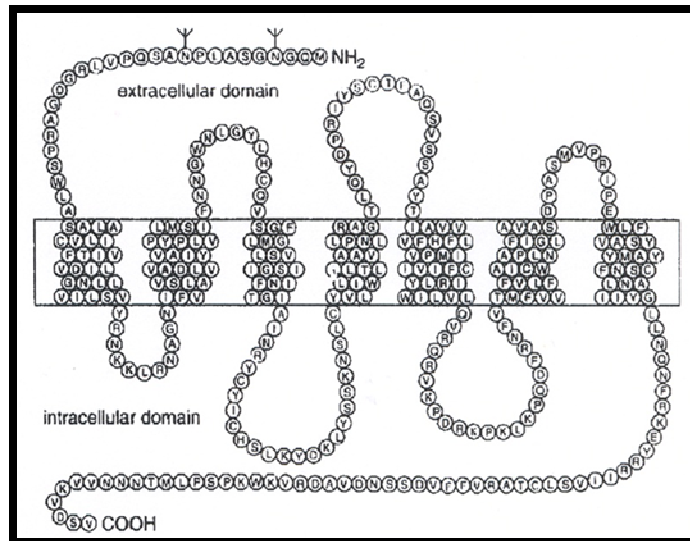
Figure 4: The membrane receptor MT1 (and likewise MT2) is a member of the seven transmembrane G protein-coupled receptors (source: [59])

MT1 and MT2 share high (overall ~55%) homology in their amino acid sequences. MT1 consists of 350 and MT2 of 362 amino acids, resulting in a predicted molecular mass of ~40 kDa for the unglycosylated MT1 ([56]). However, G-protein coupled receptors are almost always glycosylated, which explains their hydrophobic characteristic. Indeed, MT1 and MT2 possess glycosylation sites in the N-terminal region (see figure 4) and furthermore contain palmitoylatable cystein residues in the fourth intracellular loop of either receptor ([60]). The actual molecular weight of the MT1 (and the MT2) receptor is therefore most likely higher than 40 kDa. Indeed, a molecular weight of ~60 kDa was demonstrated by western blotting in MT1 transfected COS cells by Brydon and coworkers ([61]) using a self-made anti-C-terminus antibody. The C-terminal domains of MT1 and MT2 bear putative phosphorylation sites for protein kinases A and C as well as casein kinases 1 and 2 ([44]). While lipid anchor and the C-terminal tail cannot modify ligand affinity, they are required for G protein interaction ([60]). Furthermore, the phosphorylation sites are required for internalization, most likely via phosphorylation and β-arrestin binding ([9]; [54]; [62]).

Binding of the ligand melatonin results in receptor-mediated G-protein activation. The MT1 receptor can interact with different G-proteins like G_i, G_0, G_q, G_z, G_{12}, G_{13}, G_{14} or G_{16}, while MT2 interacts with G_i, G_0, G_z, G_{12} and G_{14}. In principle, the interaction with the

various G-proteins is possible in any cell type, but it depends both on how strong the melatonin receptor is expressed in the particular tissue and on the availability of the G-proteins in the cell type ([63]).

G-protein activation then leads to activation/modulation of a variety of signal-transduction pathways (see Figure 5). For MT1, this includes inhibition of the forskolin-stimulated cAMP formation, activation of protein kinase A and phosphorylation of the cAMP-responsive element binding. In addition, MT1 can activate mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2), the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) and c-Jun N-terminal kinase (JNK). In general, MT1 and MT2 activate very similar G-protein-dependent signalling pathways and only few subtype-specific differences have been reported, such as the inhibition of cGMP formation through the soluble guanylyl cyclase pathway, by MT2, but not MT1 in HEK293 cells ([64]; [62]).
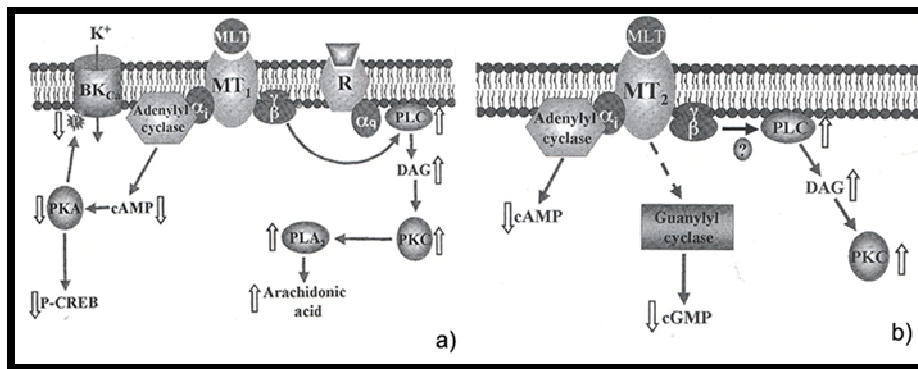
Figure 5: Both membrane receptors MT1 (Figure 1 a) and MT2 (Figure 1b) are seven-transmembrane G protein-coupled receptors. The effects of melatonin caused by binding to one of the two membrane receptors depends on the G proteins present ([65]; [59]; [63])

As suggested for other GPCRs, MT1 and MT2 do not only couple to heterotrimeric G proteins but are also physically associated with other intracellular proteins. As an example, MT1 and MT2 associate with complexes containing filamin A and insulin receptor substrate 4 (IRS4). These molecules might be important for intracellular trafficking and/or signalling. In addition, the C-terminal extremity of the MT1 receptor was shown to interact with Multi-PDZ Domain Protein 1 (MUPP1). The interaction with MUPP1 has been shown to promote coupling of MT1 to the cAMP pathway ([66]). Finally, MT1 and MT2 can form homo- and heterodimers. Thereby, MT_1/MT_2 heterodimer and MT1 homodimer formation is more likely than MT2 homodimer formation ([67]), suggesting that MT_2 may be preferentially engaged into heterodimers in cells co-expressing equimolar

quantities of both receptors. Most likely, melatonin receptors heterodimerize also with other GPCRs, as demonstrated for the orphan GPR50 receptor ([68]).

Localization of melatonin receptors MT1 and MT2 in mammalians

Demonstration of melatonin receptor expression often occurs at the mRNA level by RT-PCR techniques or in situ hybridization. The reason is the lack of reliable antibodies directed against MT1 or MT2 receptors, mainly in species other than humans ([62]).

In contrast to lower vertebrates and birds, where melatonin receptors are widely distributed in the central nervous system, mammalian species have a more restricted expression pattern of the melatonin receptors. In addition, the level of expression in mammals is markedly weaker than in non-mammalian species, with the pars tuberalis of the anterior pituitary exhibiting the highest expression levels of melatonin receptors. In mammalian species, receptor subtype MT1 mRNA is expressed in the hypothalamus, including the SCN, the cerebral cortex, thalamus, hippocampus, cerebellum, cornea, and retina, while MT2 mRNA is expressed more restricted and found only in SCN, hippocampus, retina and cerebellum ([69]; [70]; [71]). Expression in peripheral tissues such as arteries and heart (MT1, MT2), lung (MT1/MT2), kidney (MT1), liver (MT1/MT2), adrenal gland (MT1), small intestine (MT2), skin (MT1/MT2) or T and B lymphocytes has been demonstrated ([72] ; [73]; [74]; [75]; [76]; [25]).

Location of melatonin receptor MT1 in humans

Due to the fact that some antibodies exist against human MT1, not only RNA, but also protein expression of this human receptor has been studied in several tissues. The localization of the human MT1 receptor protein was confirmed in the SCN, hippocampus, nucleus accumbens, amygdala, substantia nigra, hypothalamus and cerebellum, by immunohistochemistry or immunoblotting studies using specific antibodies ([77]; [78] ; [79]; [80]; [81]; [82]). In addition, MT1 was detected by immunohistochemistry in various areas of the eye ([83]; [84]).

With respect to peripheral tissues, MT1 protein expression was confirmed in the pancreas ([85]), in the gallbladder epithelium ([86]), in normal breast tissue and in ductal carcinoma ([80]; [61]; [87]; [88]) as well as in human breast cancer cells MCF-7 ([89]). MT1 protein expression was shown in human prostate tissues of cancer patients ([90]; [31]) and in coronary arteries of humans ([91]). Finally, MT1 protein was demonstarted in the placenta and trophoblast-derived cell lines ([21]; [22])

These studies are summarized in Table 1 that also lists the antibodies and techniques used. It should be mentioned that not all of the investigators characterized the protein detected by the applied anti-MT1 antibody in westernblot experiments before application

in immunohistology/immuofluorescence microscopy (([78]); [83]; [84]; [85]; [86]; [80]; [87]; [88]; [22]; [92] [90]; [93]). From those articles that presented western blots it must be concluded that different anti-MT1 antibodies can detect proteins of variable molecular weight. Lanoix and coworkers detected a band at 40 kDa using the antibody sc-13179 directed towards the N-terminal region of MT1 ([21]; [22]). Ekmekcioglu used for his western blot analysis the antibody from CIDtech Research Inc. Toronto, Canada (that does no longer exist) and reported a band at 39 kDa ([91]). Other groups used the same antibody from CIDtech Research Inc and all reported one major band of 37 kDa by western blot ([81]; [89]; [86]). Xi and collegues detected two bands at molecular weights of 66 kDa and 37 kDa using antibody TIL3 directed against a sequence in 3rd intracellular loop of MT1, but the 66kDa band could not be blocked with anti TIL3 peptide and therefore was suggested to be caused by cross reaction with another protein ([92]). Also Song et al. used the peptide sequence TIL3 corresponding to the 3rd intracellular loop of MT1 and detected bands at 37 kDa and 66 kDa in different tissues. Since only the 37 kDa band was blocked in the presence of peptide TIL3, the 66 kDa protein was suggested to represent a cross-reacting protein ([77]). In contrast to these three antibodies detecting proteins of about 40 kDa, ([61]) generated an antibody that detected mainly a 60 kDa protein in CHO cells transfected with MT 1 cDNA.

Table 1: Survey of MT1 expression in human tissue at mRNA or protein level. The groups used different antibodies for detection of MT1 on the protein level via western blot or immunohistochemstry: blue: peptide 536; orange: antibody from CID tech Research Inc.; yellow: TIL 3 ([77]); green: antibody from Abcam (Cambridge, UK); violet: sc-13179; red: The peptides used for the antibody generation are located at 2nd intracellular loop and in the carboxy-terminus ([93]).

Tissue	mRNA	Protein	Molecular Weight detected by western blot	Antibodies	Company	References
SCN	x					
Hippo-campus		x	~ 60 kDa	peptide 536 against the C terminus of the human membrane receptor MT1 (AC-YKWKPSPLMTNNNV VKVDSV-COO$^-$)	([61])	([78])

Table 1 continued

Tissue	mRNA	Protein	Molecular Weight detected by western blot	Antibodies	Company	References
Central dopa-minergic system	x	x		Anti human MT1 antibody	CID tech Research Inc., Cambridge, Ontario, Canada	([81])
Cere-bellum	x					([94])
Hypo-thalamus		x		TIL 3 Peptide against the 3rd intracellular loop of the human MT1 receptor residues 226-238;		([77])
Retina		x		Polyclonal anti MT1 antibody against the 3rd intracellular loop of the human MT1 receptor residues 226-238;		([83])
Cornea		x	~ 60 kDa	peptide 536 against the C terminus of the human membrane receptor MT1 (AC-YKWKPSPLMTNNNV VKVDSV-COO⁻)	([61])	([84])
Pancreas		x		Polyclonal anti human MT1 antibody (type 1A, ab13036)	Abcam (Cambridge, UK)	([85])
Gall-bladder	x	x		Polyclonal anti human MT1 antibody	CID tech Research Inc., Cambridge, Ontario, Canada	([86])
Immune system	x					([95]; [96])
Breast		x	~ 60 kDa	peptide 536 against the C-terminal sequence in the MT1 protein (Ac-YKWKPSPLMTNNNV VKVDSV-COO⁻)	[61]	([80])
		x		Polyclonal anti human MT1 antibody	Abcam (Cambridge, UK)	([87])
	x	x	37 kDa	Anit human MT1 antibody	CIDtech Research Inc., Cambridge, Ontario, Canada	([89])

Table 1 continued

Tissue	mRNA	Protein	Molecular Weight detected by western blot	Antibodies	Company	References
	x	x		Polyclonal anti human MT1 antibody	Abcam (Cambridge, UK)	([88])
Myo-metrium	x					([97])
Placenta	x	x	40 kDa	polyclonal anti human MT1 antibody (sc-13179)	Santa Cruz Biotechnology, Santa Cruz, CA, USA	([21])
	x					([20])
		x			Abcam (Cambridge, MA, USA)	([22])
Prostata		x		Polyclonal anti human MT1 antibody	CIDtech Research Inc., Cambridge, Ontario, Canada	([90])
	x	x	37 kDa, 60 kDa	TIL 3 Peptide against the 3rd intracellular loop of the human MT1 receptor residues 226-238;	([77])	([92])
Coronary arteries	x	x	39 kDa	Polyclonal anti human MT1 antibody	CIDtech Research Inc. Toronto, Canada	([91])
Bone	x					([98])
NIH3T3 cells		x		133-146 (NH$_2$-LKYDKLYSSKNSLC-COOH) 341-350 (NH$_2$-NNVVKVDSV-COOH). The peptides of the antibody are located at the 2nd intracellular loop and in the carboxy-terminus.		([93])

2.4 Human placenta

2.4.1 Structure and morphology of the human placenta

The placenta consists of the three main layers chorionic plate, chorionic villi and the basal plate that is interacting directly with the endometrium of the mother. It forms a temporary

organ in the human uterus connecting mother and fetus (see Figure 6). The most important structures of the human term placenta are the chorionic villi with their terminal branches that are in contact with the blood of the mother and provide the fetus with nutrients and oxygen. In addition, chorionic villi synthesize various molecules and hormones necessary to avoid an abortion. The umbilical cord is the connection between placenta and fetus inserts into the third layer the chorionic plate ([99]; [100]).
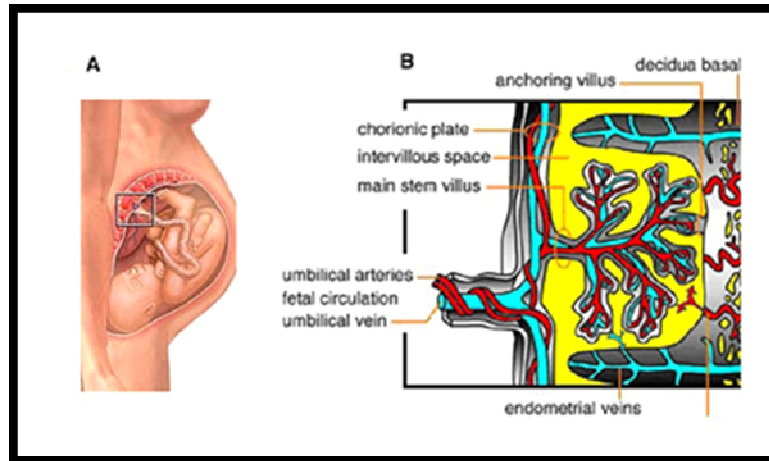
Figure 6: A: The placenta located in the human uterus is a temporary organ connecting mother and fetus via the umbilical cord

B: The placenta consits of the three main layers chorionic plate, chorionic villi and basal plate. The chorionic villi are responsible for the exchange of nutrients and gas and production of hormones (Source: [100])

Chorionic villi have a stromal core containing fibroblasts, macrophages and fetal capillaries are covered by trophoblast cells. Division, differentiation and fusion of mononuclated trophoblast cells (cytotrophoblast) result in the development of a multinucleated cell layer known as the syncytiotrophoblast (STB).

While the basal side of the STB faces the stromal core of the villi, the apical side is directed towards the intervillous space which forms the connection to the maternal blood. The side of maternal-fetal exchange is known as "vasculo-syncytial membrane"; here STB and fetal capillaries are in close contact and are only separated by a basal laminar ([99]; [100]).
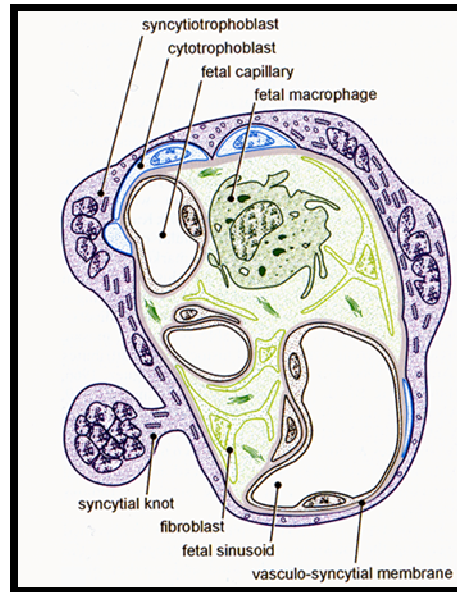
Figure 7: Terminal chorionic villous (cross section): the chorionic villi are covered by syncytiotrophoblast cells (STB). These differentiate from underlying cytotrophoblast (CTB). The stromal core of chorionic villi contains macrophages, fibroblasts and fetal capillaries ([99])

2.4.2 The most important functions of the human placenta

Five days after fertilization the early placenta invades into the endometrium to ensure implantation and placentation avoiding abortion. The fully developed placenta is not only important for maternal-fetal nutrient and oxygen exchange but also for the disposition of fetal waste products. In addition, endogenous compounds including growth factors, hormones and cytokines are synthesized in this organ. Antioxidative systems provided by the healthy placenta protect the fetus from pathological and immunological attack as well as ROS and RNS produced in the placenta ([99]; [100]).

2.4.3 The pathogenesis of preeclampsia

For 5 % of all pregnancies the occurrence of preeclampsia has been reported. Typical symptoms including hypertension and proteinuria, might be caused by maternal endothelial dysfunction ([101]; [102]). Defects in endothelial cell activation and permeability are -among many other reasons- caused by increased circulating ROS levels produced in the abnormal placenta ([103]).Therefore, oxidative stress is related to the patghogenesis of preeclampsia (PE; [102]). Preeclampsia is thought to be responsible for the developement of cardiovascular diseases later in life. Severe consequences of this disease are obstetric mortal and morbid incidences ([104]; [102]). Beside the maternal

syndroms preeclampsia can cause growth restriction of the fetus. Although the reason of the devolpment of pre-eclampsia (PE) is still unknown evidences indicate involvement of multiple genetic and immunologic factors ([101]).

2.4.4 In vitro model of trophoblast function: The immortalized chorio-carcinoma (BeWo) cell line

The immortalized choriocarcinoma BeWo cell lines ([105]) is a common used *in vitro* model for studying function of the human placenta especially, transport of nutrients and xenobiotics across trophoblast cells ([100]; [106], [107]; [108]; [109]; [110]; [111]; [112]). BeWo cells form a polarized cell monolayers and synthesize placental hormones like hCG and progesterone. ([113]; [114]).

2.5 The role of melatonin in human pregnancy

2.5.1 The concentration of melatonin in the serum of the mother

After 24 weeks of pregnancy there is a significant augmentation in the nocturnal serum level of melatonin and a further elevation occurs after 32 weeks of pregnancy in comparision to non-pregnant woman. Melatonin levels are to non-pregant state already 48hours after puerperium ([115]; [116]). Similar alterations in melatonin concentrations during pregnancy were observed in rats. These changing melatonin levels are suggested to be caused by unknown placental hormones influencing secretion of the maternal pineal gland ([19]).

2.5.2 The passage of melatonin thorough the placenta and the production of melatonin by the fetus

Not only humans, but also other species demonstrate passaging of melatonin through the placenta, indicating that melatonin levels in the fetus are of maternal origin ([117]; [118]; [119]). Although plasma melatonin levels in rat fetus decreased after pinealectomy of the maternal rat ([120]) and neonates are not able to produce melatonin until the 4th month ([121]; [122]), it is nevertheless postulated that the fetus is able to produce melatonin itself. This is indicated by higher levels of this hormone in umbilical arteries compared to corresponding veins ([115]) and the rat fetal brain can to synthesize melatonin ([122]).

2.5.3 The influences of MT onto the fetus

The clear function of melatonin in the fetus is unknown, but published data indicate that melatonin regulates the rapid eye movement of the fetus and fetal gonadal growth via melatonin-induced modulation of fetal clock gene functions ([123]; [124]; [125]). This is reported to be caused by interaction with the MT1 in the fetal SCN ([126]). Furthermore, circadian rhythm is thought to be entrained to the fetus via melatonin and it therefore seems that melatonin synchronizes maternal and fetal physiology ([127]; [128]; [129]; [130], [131]; [132]; [133]). However, no proof of this hypothesis is available as high doses of melatonin administred to pregnant rats did not effect fetal growth, viability or morphology. ([134]).

2.5.4 The mebane receptors MT1 and MT2 in placenta and uterus

Both melatonin membrane receptors MT1 and MT2 have been identified in first trimester as well as term placenta either at the mRNA or the protein level, respectively. ([20]). In term placenta, these receptors are suggested to be located in cytotrophoblast, syncytiotrophoblast (STB), in endothelial cells of fetal capillaries and in the mesenchymal core of the chorionic villi ([22]). These results are confirmed as MT1 and MT2 have also been identified in isolated, *in vitro* differentiated villous trophoblasts of term placenta. JEG-3 and BeWo cells, derived from placental choriocarcinoma, also express both membrane receptors for melatonin ([22]).

Melatonin seems to be involved in the feto-placental development since melatonin receptors are not only expressed in human as well as in rat placenta, but melatonin is also able to augment the human chorionic gonadotrophin (hCG) level and to downregulate the mRNA expression of placental lactogen II in the rat placenta ([135], [20]; [22]).

2.5.5 The influences of oxidative stress and melatonin on pregnancy

During pregnancy, an increase in metabolism is required. A higher demand of oxygen subsequently increases the production of ROS since mitochondria in first trimester placenta are known to produce this source of oxidative stress ([136]). Under normal physiologic conditions of the pregnancy, lipid peroxides derived from the high content of polyunsaturated fatty acids in the placenta in combination with ROS are controlled by the placental anti-oxidative enzymes superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, glutathione S-transferase and glucose-6-phosphate dehydrogenase ([137]). In addition, melatonin is involved in

oxidative stress reduction, indicated by observed higher plasma melatonin levels in pregnant women. The increase in melatonin levels may be due to its production in the placenta indicated by the detection of melatonin synthezing enzymes (NAT and HIOMT) in this organ. ([22]; [20]). Interestingly, spontaneous abortion may be correlated to placental oxidative stress as in pineaolectomized rats melatonin levels were decreased and the incidence of abortion was observed to occur more frequent ([138]; [139]; [140];.[141]). Therefore, melatonin is indicated to prevent abortion due to its anti-oxidative properties. Protection of the fetus and maintenance of pregnancy by melatonin is further accomplished by immunomodulatory function und stimulation of progesterone production in the ovary, respectively ([142]; [19]). Furthermore, melatonin is reported to elevate levels of the placental anti-oxidative enzymes, the manganese superoxide dismutase (Mn-SOD) and catalase, and therefore improve its efficiency ([143]).

2.5.6 Preeclampsia and the involvement of melatonin

That preeclampsia is related to higher oxidative stress is indicated by studies showing higher lipid peroxidation and decreased efficiency of anti-oxidative systems in preeclampsia affected women ([144]; [145]; [146]; [147], [148]; [149]). Evidence for the involvement of melatonin in the progress of this disease is given by the detection of lower nocturnal melatonin levels in PE pregnancies ([115]). Moreover, it was observed that preeclampsia can be prevented by the administration of anti-oxidants like vitamin C and E ([150]). Although the clear mechanism of melatonin-effected PE prevention is still unknown ([19]; [151]), it is postulated that melatonin increases the levels of anti-oxidative enzymes and therefore protects radical induced injury of the placenta.

To summarize, the existence of melatonin synthesizing enzymes and receptors in the human placenta indicates a critical role of this hormone for proper placental function. Available literature supports direct (anti-oxidative effect) or indirect effects (via receptors) of melatonin. Nevertheless, further investigations are necessary to clarify involvement of melatonin in the pregnant disease preeclampsie.

3 Aims

Expression of MT1, MT2 and other melatonin receptors has been demonstrated in the human placenta ([20], [22]), especially in the syncytiotrophoblast (STB) representing the interface between maternal blood and placental tissue. Based on the literature, it can be speculated that maternal and/or placental derived melatonin via receptor-mediated processes plays an important role in the regulation of trophoblast development (e.g. proliferation, differentiation, and apoptosis; ([152]; [153]) and endocrine function (e.g. hormone production; ([135]; [20]; [22]). Furthermore, an application of the radical scavenger melatonin in the prevention or treatment of preeclampsia, that involves excessive free radical production, has been suggested ([36]). Application of melatonin in the course of pregnancy should, however, imply knowledge of the effects of melatonin mediated via high-affinity placental MT1 or MT2 receptors. Currently, receptor-mediated effects of melatonin in the placental syncytiotrophoblast, have not been investigated systematically. Such investigations require, firstly, an appropriate *in vitro* system. BeWo choriocarcinoma cells derived from placental trophoblast cells ([105]) represent a candidate cell line for such investigations. They endogenously express the high-affinity receptors MT1 as well as MT2 ([21]), and they are a characterized *in vitro* system for trophoblast cells ([100]). Secondly, BeWo cells with transiently or permanently elevated expression levels of MT1 or MT2 would be a valuable tool to specifically address the function(s) of the receptor under investigation and allow discrimination from other melatonin-associated processes. Thirdly, specific antibodies against melatonin receptors are indispensable for better characterization of the proteins with respect to e.g. cellular steady-state distribution and redistribution/cellular fate upon ligand binding. For human MT1, generation and application of various antibodies have been described (see table 1, introduction), but in summary, only a limited number of antibodies against MT1 is available, the majority of them being not well characterized. In order to investigate MT1 function in trophoblast-derived BeWo cells a lipid-based transfection protocol was optimized for BeWo cells and the RT-PCR protocols as tools for overexpression of MT1 and for detection of MT1 mRNA were established, respectively. Furthermore, transient transfection of BeWo cells with human MT1 cDNA was cloned into the mammalian expression vector pcDNA 3 tagged with either flag or triple HA peptides or the green fluorescent protein (GFP), including verification of MT1-mRNA (over)expression by RT-PCR protocols. Following successful transient transfection, stably MT1-transformed cell lines should be generated by appropriate antibiotic selection. Moreover, various anti-MT1, anti-flag-tag or anti-HA-tag antibodies were characterized in western blotting experiments on lysates of MT1 transfected or parental BeWo cells.

4 Materials and Methods

4.1 Cell culture

Cell culture

The culturing of cells has to be done under sterile conditions. This implicates to manipulate cells in the laminar flow, to clean surfaces in contact with the culture flasks with 70 % ethanol and to use sterile materials and solutions (e.g. heat-sterilized, autoclaved, sterile-filtered). If not mentioned otherwise, all used media and solutions are pre-warmed to 37 °C.

The incubating conditions for cells are 5 % CO_2 in the atmosphere, a humidity of 95 % and a temperature of 37 °C.

Transfection

This method is used to introduce a DNA sequence of interest into eucaryotic cells. Several techniques exist. FuGENE® HD transfection reagent (Roche Diagnostics, Germany) is a blend of lipids and other components supplied in ethanol that forms a complex with DNA and transports the complex into the animal cell. The transfected genetic material is then transiently (for a short period) expressed by the cell. Stable transfection is accomplished by cotransfection of a gene that introduces a selectable advantage into the cell (e.g. the neomycin resistance gene encoding a gene product that neutralizes the toxin Geneticin (G418).

Cells that have, by chance, integrated the foreign material into their genome, can proliferate in the presence of the toxin, while all others die. In any way, the resulting "over-expression" of a protein of interest allows for investigation of its functionality and localization. In case, no specific antibodies against the expressed protein are available, it is possible to link the cDNA of the protein under investigation to short peptide tags in eukaryotic expression vectors. The most common tags are the FLAG tag (an octapeptide sequence, YPYDVPDYA, 1102 Da) and the hemagglutinin (HA) tag (a nonapeptide sequence, DYKDDDDK, 1012 Da). Against both peptides, commercial antibodies are available, allowing the detection of tagged protein by either indirect immunofluorescence (IF) or western blotting.

Another important tag is the living-color protein GFP (green fluorescent protein). It is derived from the jellyfish Aequorea Victoria. Upon fusion of the GFP-tag with the protein of interest, the protein can be detected by direct IF (in living or fixed cells; [154]).

4.1.1 Materials

- Osmometer (Osmomat 030-D, Gonotec)
- pH meter (827 pHLab, Metrohm)
- Stirrer
- Incubator (Cytoperm2/Heraeus) set to 5 % (v/v) CO_2, 95 % (v/v) humidity and 37 °C temperature
- Laminar Flow (Herasafe KSP 12, ThermoScientific)
- Centrifuge (Rotixa/RP, Hettich) used at 200 x g (i.e. 1000 rpm)
- Inverted Microscope (Olympus, CK2), equipped with 10 x, 20 x and 40 x objectives (total magnification: 100 x – 400 x)
- Nano drop (peqlab Biotechnologie GmbH ND-1000)
- Coulter Counter (Beckman Coulter™)
- Fluorescence microscope (Zeiss Observer Z 1)
- Waterbath (GFL 1083) set to 37 °C, filled with A.b id. treated with Aqua Resist (VWR Int. 462-7000)
- Kryostorage system (- 196 °C, K8, Kendro)
- 50 ml centrifuge tubes, sterile (TPP 91051)
- 15 ml centrifuge tubes, sterile (IWAKI Code: 2323-015)
- 1.5 ml cryogenic vials, sterile (Nalge Company 5000-1020)
- 25 cm² canted neck tissue culture flasks, sterile (IWAKI 3103-025)
- 75 cm² canted neck tissue culture flasks, sterile (IWAKI 3110-075)
- 6 well plates (Falcon 3046; IWAKI 3810006),
  12 well plates (IWAKI 3815012)
  24 well plates (Costar 3524; IWAKI 3820024)
  10 cm² plates (Falcon™ 351008)
- 230 mm glass pasteur pipettes, sterile (VWR 612-1702)
- Gloves (e.g. Latex-gloves Peha-soft; Hartmann 942 161)
- Syringe
- Sterile syringe filters, 25 mm, 0.2 Micron (IWAKI 2052-025)
- Filter 40 µm Nylon (BD Falcon 7186958)
- Tips for pipettes
- Micropipetts

4.1.2 Reagents

- KCl (Merck 4936.1000)
- NaCl (Merck 1.06404.1000)
- KH_2PO_4 (Merck 1.04873.1000)
- Na_2HPO_4. $2H_2O$ (Merck 1.06580.1000)
- Aqua bidestillata
- PCR water (Gibco 10977-035)
- Dulbecco´s modified eagle medium (DMEM; Sigma D-5796; 500 ml) sterile, containing 4500 mg/l glucose, L-glutamine, and sodium bicarbonate without sodium pyruvate, liquid, cell culture tested), stored at 4 ℃
- Fetal calf serum (FCS; Gibco/Invitrogen 10270-106 or PAA A15-15; 500 ml) sterile, stored in ready-to-use aliquots (50 ml) at-20 ℃
- RPMI 1640 (PAN BIOTECH GmBH P04-16520; 500 ml), sterile, stored at 4 ℃
- 200 mM Glutamine (GlutaMAX-I Gibco/Invitrogen 35050-038; 100 ml), sterile, stored in ready-to-use aliquots (5.5 ml) at-20 ℃
- Liquid Antibiotic Mixture (PSN, Gibco/Invitrogen 15640-055, 100 ml, sterile, containing 5 mg/ml Penicillin, 10 mg/ml Streptomycin and 10 mg/ml Neomycin, stored in ready- to-use aliquots (5.5 ml) at-20 ℃
- Dimethyl sulphoxide (DMSO; Sigma D2650, 5 x 5 ml), sterile stored at rt
- 10 x Trypsin/EDTA solution, (Gibco/Invitrogen 15400-054, 100 ml), sterile-filtered, containing 0.5 g porcine trypsin, 0.2 g EDTA x 4 Na per liter of 0.9 % NaCl, stored at-20 ℃
- Phenol red (Sigma P3532)
- Geneticin (G418, Gibco, 10131-027, 50 mg/ml stock solution), sterile, stored at 4 ℃

4.1.3 Buffers and media

Heat inactivation and aliquoting of FCS

- Incubate FCS for 30 min at 56 ℃ (= heat inactivat ion); Sway the medium every few minutes
- Transfer the medium to the laminar flow
- Aliquote FCS in 50 ml tubes
- Freeze and store at -20 ℃

Phosphate- buffered solution, PBS (1 x), sterile

Dissolve the following salts in 800 ml A.bid.

- 0.20 g KCl, final concentration 2.7 mM
- 8.01 g NaCl, final concentration 137 mM
- 0.21 g KH_2PO_4, final concentration 1.5 mM
- 1.44 g Na_2HPO_4 x 2 H_2O, final concentration 8.1 mM
- Adjust pH to 7.4 with 0.1 N NaOH or HCl
- Adjust volume to 1000 ml with additional A.bid.
- Measure and note osmolarity (should be 270-300 mOsm kg)
- Sterilize by autoclaving
- Store at rt

Complete culture medium for BeWo cells and MDCK II cells = Complete DMEM

- 500 ml DMEM, high glucose, sterile
- 50 ml FCS, sterile (final concentration 9 % (v/v))
- 5.5 ml 200 mM Glutamine (final concentration 2 mM)
- 5.5 ml liquid PSN (final concentration 50 µg/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml neomycin)
- Store at 4 ℃

Complete culture medium for HOS and MG 63 cells = Complete RPMI 1640

- 500 ml RPMI 1640
- 50 ml FCS, sterile (final concentration 9 % (v/v))
- 5.5 ml 200 mM Glutamine (final concentration 2 mM)
- 5.5 ml PSN (final concentration 50 µg/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml neomycin)
- Store at 4 ℃

Complete DMEM + G418 = Complete selection medium

- To 500 ml complete DMEM add G418 (stock 50 mg/ml)
  (final concentration 0.25 mg/ml for BeWo and 0.5 mg/ml for MDCK II)

Transfection medium

- To 100 ml DMEM without FCS
- Add 1 ml Glutamax

- Store at 4 ℃

0.5 % (w/v) phenol red solution, sterile

- Dissolve 50 mg phenol red in 10 ml A.bid.
- Sterililize by filtration using a syringe and a disposable filter
- Store at 4 ℃

Trypsin/EDTA working solution (1x), sterile

- 100 ml 10 x Trypsin/EDTA solution, sterile, (final concentration 0.05 % and 0.02 % (w/v)) , respectively
- 2 ml 0.5 % (w/v) Phenol red solution, sterile, (final concentration 0.001 % (w/v))
- Add PBS, sterile, to 1000 ml
- Aliqote sterile in 50 ml tubes and store working aliquot at 4 ℃
- For long term storage, keep aliquots at-20 ℃

Freezing medium for BeWo and MDCK II cells

- Complete DMEM (4.1.3)
- 5 ml FCS (final concentration 19% (v/v))
- 1 tube (5 ml) DMSO, (final concentration, 10 % (v/v))
- Store at 4 ℃

Freezing medium for HOS cells and MG 63 cells

- 40 ml complete RPMI 1640 (4.1.3)
- 5 ml FCS (final concentration 19 % (v/v))
- 5 ml DMSO (final concentration, 10 % (v/v))
- Store at 4 ℃

Coulter® Isoton® II dilutent

(REF 8448011)

FuGENE® HD transfection reagent

(Roche 04883560001)

4.1.4 Cell lines

BeWo cells (clone c24)

The BeWo cell line represents a model for placental trophoblast with many properties of human cytotrophoblast ([155]; [156]). It originates from a human choriocarcinoma ([157]). The subclone b24, derived from the parental line BeWo, shows many morphological and biochemical characteristics typical for placental trophoblasts ([158]; [109]; [159]) and was kindly provided by Alan Schwartz (School of Medicine, Washington University, St. Louis, MO) and Arie van der Ende (laboratory of Cell Biology; Medical School, Univ. Utrecht, The Netherlands).

BeWo cells (ATCC cell line)

Cells were kindly provided by Ao. Prof. Dr. Martin Knöfler (Department of Obstetrics and Gynecology/MUW/Vienna).

MDCK II (ATCC cell line)

Madine-Darby canine kidney cells II (MDCK II) were isolated from the kidney tissue of a cockerspaniel by SH Madin and NB Darbin ([160]). Strain II of the MDCK II cells forms epithelial monolayers ([161]) and presents a model to study protein traffic in epithelial cells ([162]). The expression of MT1 in this cell line has not been investigated at mRNA or protein level so far, however melatonin binding to the cells was not detected ([163]), indicating the absence of a high affinity binding site in MDCK II cells.

HOS and MG63

are human osteosarcoma cell lines. The HOS cell line derives from a caucasian woman, whereas the MG 63 cells origins from an Caucasian man (**http://www.lgcstandards-atcc.org**). Both, MG 63 and HOS cells, express MT1 mRNA and were used as positive control ([98]). The cells were kindly provided by Ao. Prof. Dr. Theresia Thalhammer (Department of Pathophysiology and Allergy Research/MUW/Vienna).

4.1.5 Plasmids:

pGFP-N3 (Clontech)

The plasmide pGFP-N3 (size: 4724 bp) was obtained from Ao. Prof. Dr. Martin Knöfler (Department of Obstetrics and Gynecology/MUW/Vienna). Transfection of the GFP

expression vector can be used to control the effectiveness of transfection-conditions by investigating GFP expression by direct fluorescence microscopy ([154]).

MT1- GFP-pcDNA3

This plasmid was kindly provided by Dr. Paula Witt-Enderby, Division of Pharmaceutical Sciences, School of Pharmacy, Pittsburgh, PA, USA. The cDNA for human MT1, with GFP located on the C-terminal site of MT1, was cloned into the plasmid pcDNA3 ([60]).

MT1- flag-pcDNA3

The plasmid was received from Dr. Perry Barret, Molecular Endocrinology Group, Rowett Research Institute, Aberdeen, Scotland, UK. The plasmid pcDNA3 contains the human MT1 with a flag epitope sequence introduced directly after the start codon ([164]).

MT1- HA-pcDNA3

The plasmid was received from Dr. Tarja Kokkola, (Institute of Human Genetics; University Medical Center Hamburg Eppendorf; Hamburg; Germany). Human MT1 cDNA was tagged with a triple hemagglutinin (HA) epitope directly after the N-terminal start codon of MT1 and was inserted into the pcDNA3 vector ([165]).

The plasmids MT1-GFP-pcDNA3 and MT1-flag-pcDNA3 have been transformed into competent bacteria (E. Coli), amplified and purified using Plasmid purification kit (Quiagen Plasmid Maxi kit) during a research stay of Miroslava Benova, Comenius University of Bratislava, in the laboratory of Isabella Ellinger.

The plasmid MT1-3xHA-pcDNA3 has been amplified and purified in the same way by Mehdi Ramezanian in the laboratory of Isabella Ellinger during his diplomathesis ([166]).

4.1.6 Methods

Thawing of MDCK II cells and BeWo cells

- Store cells at-196 ℃ in the liquid nitrogen storage container
- Thaw cells rapidly at 37 ℃ (by keeping the tubes in the hand)
- Transfer the cells to the laminar flow
- Put 5 ml complete DMEM to a centrifuge tube
- Add the thawed cells using a Pasteur pipette
- Pellet cells by centrifugation at 200 x g for 5 min at rt

- Carefully aspirate the supernatant
- Add 5 ml DMEM to the centrifuge tube
- Re-suspend cells by pipetting up and down twice
- Transfer the suspension to 1 x 25 cm^2 flask
- Examine the cells by light microscopy to control their appearance and note abnormities (e.g. infections)
- Place cells in the incubator at 37 ℃

Thawing HOS and MG 63 cells

Same as for MDCK II and BeWo cells but replace complete DMEM with RPMI 1640 (4.1.3)

Feeding BeWo and MDCK II cells

- Examine the cells by light microscopy to control their appearance and other abnormities (e.g. infections, detachment)
- Transfer the cells to the Laminar Flow. Always work with only 1 cell line at the time to avoid cross-contamination
- Aspirate the medium from the flasks
- Wash the cells with PBS: add 10 ml of PBS to the 75 cm^2 flask and 5 ml of PBS to the 25 cm^2 flasks
- Sway carefully
- Aspirate the PBS
- To feed the cells, add complete DMEM: 5 ml to each small flask (25 cm^2) and 10 ml to each large flask (75 cm^2)
- Return the flasks in the incubator at 37 ℃

Feeding MG-63 cells and HOS cells

Follow protocol for feeding BeWo and MDCK II cells but complete DMEM is replaced with complete RPMI 1640 (4.1.3).

Freezing of MDCK II, BeWo, HOS and MG 63 cells

- Culture cells in 1 x 75 cm^2 flask to sub-confluency (should be 80 %)
- Transfer healthy cells to the laminar flow
- Aspirate the culture medium
- Wash cells with 10 ml PBS (75 cm^2 flask)
- Aspirate the PBS

- Add 1 ml Trypsin-EDTA working solution
- Incubate for about 15 min (depends on cell line and trypsin-activity)
- Examine the cells by light microscopy; cells should round up and detach from the surface
- Upon detachment, add 10 ml culture medium
- Resuspend cells by carefully pipetting up and down twice
- Transfer cell suspension to centrifuge tube (15 ml)
- Pellet the cells by centrifugation at 200 $x\,g$ for 5 min at 4 °C
- Discard the supernatant
- Place the tubes with cells on ice
- Add 10 ml freezing medium for BeWo and MDCK II cells (4.1.3), or HOS and MG 63 cells (4.1.3), respectively (precooled to 4 °C)
- Re-suspend the cells by carfully pipetting up and down twice
- Transfer cell suspension to 10 cryotubes (1 ml/tube)
- Place tubes on ice in a small box
- Transfer box to -80 °C
- The next day transfer tubes to-196 °C (liquid nitr ogen storage container)

Passaging (Splitting) of MDCK II, BeWo, HOS and MG 63 cells

- Transfer healthy cells to the laminar flow
- Aspirate the medium
- Wash cells twice with 5 ml PBS to remove the debris, dead cells and trypsin inhibitor contained in the medium
- Add Trypsin-EDTA working solution (0.5 ml/25 cm^2; 1 ml/75 cm^2)
- Place in the incubator for about 15 min at 37 °C (the incubation time depends on the cell line, trypsin activity and degree of confluency)
- Examine the cells by light microscopy; cells should round up and detach from the substrate
- When cells are detached, add 5 ml of respective complete culture medium
- Resuspend the cells by carefully pipetting up and down twice
- Transfer aliquots of the cell suspension to new flasks containing the respective culture medium. Maximum splitting rate for BeWo is 1 : 5, for MDCK II 1 : 10, for HOS and MG 63 1 : 10, and add complete medium to a final volume of 10 ml/75 cm^2 or 5 ml/25 cm^2
- Return cells to the incubator

Counting the cells with the coulter counter

- Culture the cells in 1 x 75 cm^2 flask to sub-confluency (80 %)
- Follow protocol (4.1.6; „Passaging (Splitting) of MDCK II, BeWo, HOS and MG 63 cells) until cells are detached after addition of trypsin solution
- When cells are detached, add 5 ml culture medium to the flask
- Resuspend the cells by pipetting up and down twice
- Put the resuspended cells in a centrifuge tube
- Centrifuge for 5 min with 200 *x g* at rt
- Aspirate the supernatant
- Resuspend the pellet in 1000 µl complete DMEM
- Filter the suspension through a 40 µm Nylonfilter to make sure that there are no cell clots, as the coulter counter would count the clot as one cell
- Put the tube on ice
- Take a sample of 100 µl
- Dilute the sample with 10 ml isotone solution (4.1.3) and measure the suspension with the coulter counter according to the instructions of the manufacturer

Counting the cells with the hemocytometer

- Follow protocol in 4.1.6 where the coulter counter is used. Instead of measuring 100 µl of re-suspended cells, dilute an appropriate volume (e.g. 50 µl) with medium 1 : 10 (1 + 9) before pipetting an aliquote (20 µl) of the dilution on the counting chamber
- Count cells in 4 out of 16 quadrants (i.e. numbers of cells in quadrant a-d)
- Apply the following formula for determination of cells in 1000 µl cell suspension.

$$\Sigma = (a + b + c + d) \text{ cells x 2500 x 10} = \textbf{cells/1000 µl}$$

Plating of cells prior transfection with Fugene HD

- Count cells in 1000 µl cell suspension using the coulter counter or the hemocytometer (4.1.6).
- Prepare an appropriate multi well plate (6, 12 or 24 well plate) by adding complete DMEM to the wells (2, 1 or 0.5 ml), respectively
- Calculate the number of cells to be seeded /well
- Cell numbers/well were varied
  1) due to the area of wells used (6 well plate: 9.4 cm^2/1 well, 12 well plate: 3.8 cm^2/1 well, 24 well plate: 2 cm^2/1 well)

2) during optimization for the different cell strains used (BeWo, MDCK II)

- The numbers are indicated in the result section
- Add to each well the respective amount of cells
- Incubate cells at 37 ℃

Transfecting cells using FuGENE® HD Transfection reagent

The transfection was made according to the instructions of the company (Roche Diagnostics, Germany).

- Allow FuGENE® HD transfection reagent, DNA and transfection medium to adjust to rt
- Vortex the FuGENE® HD
- Determine the concentration of plasmid DNA with nanodrop
- Mix 2 µg plasmid DNA with 100 µl transfection medium (in sterile tubes)
- Add FuGENE® HD to each tube to form the transfection complex. During the optimization the optimum amount of FuGENE® HD used was varied from 3 µl-12 µl. Pipet the FuGENE® HDtransfection reagent directly into the medium containing the diluted DNA without contacting the walls of the plastic tubes
- Vortex briefly (~ 2 seconds)
- Incubate the DNA complex for 15 minutes at rt
- Get the cultured cells from the incubator. Do not remove the growth medium
- Add the transfection complex to the cells in a drop-wise manner
- Incubate the cells for 24 hours at 37 ℃
- Determine expression of protein of interest by an appropriate method
    1) Following transfection with pGFP-N3, measure the GFP-protein expression by microscopic examination (4.2; time points 24 h and 48 h). Transfected cells should exhibit green fluorescence due to expression
    2) Following transfection with plasmids containing human MT1 cDNA evaluate expression either at the mRNA level by RT-PCR (4.4; 4.5; 4.6) or analyze expression by SDS-PAGE and western blot experiments (section 4.7.4)

Selection and cloning of transfected cells to generate stably transfected cell lines

- Having confirmed successful transient transfection with a plasmid of interest by RT-PCR (sections 4.4; 4.5; 4.6), replace the complete medium with complete selection medium

- Wash cells every 2nd day with PBS (4.1.3) and add fresh complete selection medium (4.1.3) until the non transfected cells are dead and cell islands appear in the transfected cells (~ 10-14 days)
- Prepare sterile cloning rings. The upper part of blue tips are cutted with a razor blade thereby rings of 1 cm hight are obtained
- Sterilize them in ethanol
- Autoclave grease in a glass Petridish
- Wash cells with PBS
- Remove PBS
- Dip rings with 1 side in grease
- Place the ring with this side around a single colony (the grease should seal the interior so that the trypsin added in the next step stays in the ring)
- Put trypsin-solution into the ring
- Wait until cells detach
- Transfer cells of 1 clon into 1 well of a 24 well plate
- Expand transfected cells by transfering cells upon confluency to 12 well plates, 6 well plates and finally 25 cm^2 flasks
- Freeze an aliquote of each transfected cell clone as soon as possible and test clones for expression of protein of interest by a method of choice (e.g. SDS-PAGE and western blotting)

4.2 Microscopy

For microscopic investigations of cells, the fully motorised inverted microscope Axio Observer Z1 equipped with a HB 100 lamp, a HAL 100 lamp and an Axio Cam MRc5 (Zeiss, Germany) was used. The inverted microscope allowed for the analysis of living cells in a cell culture plate without prior fixation.

To study optimal cell densities or morphology of cells following transfection, phase contrast microscopy was applied using 10 x, 20 x or 32 x LD-A-Plan objectives (0.25 Ph 1, 0.3 Ph 1 or 0.4 Ph 1, respectively).

For examination of GFP fluorescence, living transfected cells were investigated by fluorescence microscopy using filter set 10 (Zeiss 488010-0000; Ex: BP 450-490, beamsplitter FT 510, Em: BP 515-564). Fluorescence images (GFP-fluorescence) and correlating phase contrast images were collected from different regions of the wells, using 10 x, 20 x or 32 x objectives, and stored as .tif files. For presentations, images were processed identically by Adobe Photoshop.

4.3 RNA isolation

A basic requirement for the demonstration of mRNA expression by RT-PCR experiments is the isolation of clean and intact RNA, i.e. a RNA free from nucleases, macromolecules or salts ([167]). Therefore, the solutions and materials used during the isolation have to be RNase free and it is absolutely essential to wear gloves during the entire extraction procedure to prevent any contamination with RNases.

RNA can be isolated in a single step method by an acid guanidinium thiocyanate-phenol-chloroform extraction ([168]). Numerous commercial reagents based on this process are meanwhile available, such as the solution peqGOLD TriFast™ (Peqlab), used in this study. peqGOLD TriFast™ (Peqlab) contains guanidinium thiocyanate (GTC) that lyses cells and inactivats RNases and other enzymes. Phenol contained in the reagent dissolves DNA and proteins. Addition of chloroform and subsequent centrifugation allows for separation of RNA (upper phase) from the DNA (interphase; [167]). Finally, RNA is precipitated by addition of isopropanol, washed with 70 % ethanol and dissolved in RNase and DNase free water ([167]). The RNA can then be subjected to RT-PCR reactions, after determination of its concentration, purity and intactness.

4.3.1 Materials

- Heating block (Thermomixer 5436, Eppendorf)
- Centrifuge with temperature setting to 4 °C (Eppen dorf 5415R)
- Nano drop (peqlab Biotechnologie GmbH ND-1000)
- Microcentrifuge tubes, 1.5 ml (Biozym 710310)
- Micropipettes
- Gloves (e.g. Latex-gloves Peha-soft; Hartmann 942 161)
- Safe seal tipps (1000 µl Peqlab 81-1050; 20 µl Peqlab 81-1020; 10 µl Biozym 770010)
- Ice

4.3.2 Reagents

- TriFast TM Reagent (Peqlab 30 2010)
- Chloroform (Merck 2445)
- Isopropanol (Merck 1.09634.1011)
- Ethanol (VWR Prolabo, 20821.310)

- 70 % ethanol prepared by addition of PCR water to ethanol
- PCR water DNase/RNase free (Gibco 10977-035)

4.3.3 Methods

RNA isolation

CAVE: Both phenol and GTC are harmful. Therefore, it is necessary to work in a fume hood during the whole RNA isolation, to wear gloves and protective eye glasses.

The isolation is done according to the protocol of the manufacturer.
- Grow cells to 80-90 % confluency (25 cm^2 flask)
- Wash cells in the culture flask with 5 ml ice cold PBS (2 x)
- Add 2 ml of TriFast ^{TM} Reagent (Peqlab), put immediately on ice and incubate 5 min
- Lyse cells by repetitive pipetting, transfer lysate into 1.5 ml fresh tube (1 ml/tube)
- Add 200 µl chloroform/1 ml Tri Fast ^{TM} Reagent (i.e. 400 µl per 2 ml)
- Shake samples vigorously for 15 sec
- Incubate for 10 min at rt
- Separate mixture into phenol-chloroform phase (DNA, protein), the interphase (DNA, proteins) and upper aqueous phase (RNA) by centrifugation for 5 min at 12.000 $x\,g$ at 4 °C
- Remove the upper aqueous phase containing RNA carefully
- Transfer to fresh tube (labelled)
- Centrifuge again for 5 min at 12.000 $x\,g$ at 4 °C to make sure that no interface contamination (DNA) is in the aqueous phase
- Transfer the upper phase to fresh tubes (labelled)
- Add 500 µl isopropanol/tube and precipitate the RNA by incubation for 10 min on ice
- Centrifuge for 10 min at 12.000 $x\,g$ at 4 °C
- Remove and discard supernatant (isopropanol)
- Wash the pellet with 1000 µl 70 % ethanol by pipetting up and down several times
- Centrifuge for 10 min at 12.000 $x\,g$ at 4 °C
- Remove the ethanol (thoroughly)
- Dry pellets with an opened lid in a heating block at 55 °C until ethanol evaporates (1 min, max 3 min!). Do not dry completely, otherwise RNA becomes insoluble!

- Add 20 µl PCR water/tube
- Dissolve the RNA pellet by pipetting up and down several times
- Incubate with closed lid for 10 min at 55 ℃ to dissolve the RNA
- Store RNA at-80 ℃

Determination of quantity and purity of the RNA

Concentration as well as purity of the isolated RNA are determined with the UV/VIS spectral photometer ND-1000. This spectrophotometer does not use cuvettes and requires only 1.0 µl RNA samples for measurement. RNA concentration is determined by measuring the absorbance at 260 nm. Absorptions of the sample at 280 nm as well as 230 nm are also determined to investigate the contaminations of the RNA sample with respect to protein or phenol. The ratio of absorption at 260/280 should be around 2.0 for RNA samples (for DNA the ratio is closer to 1.8). A ratio much lower than 2.0 indicates a contamination with e.g. proteins or phenol that absorb strongly at or near 280 nm.

The ratio of absorption at 260/230 is also a measure for RNA purity. Expected values are in the range of 2.0-2.2. A lower ratio than this indicates contamination with phenol.

- Measure 1.5 µl PCR water and blank the instrument
- Vortex the sample briefely
- Pipette 1.5 µl RNA sample onto the nanodrop
- Measure
- Note the concentraion and the ratios determined

(NanoDrop Technologies, Inc **www.nanodrop.com**; ©2009;
www.molcardiology.com/attachments/nanodrop.pdf)

4.4 Reverse transcription

During reverse transcription the sequence of a single stranded RNA gets reversely transcribed via the enzyme reverse transcriptase into the complementary DNA (cDNA) ([169]) being required for subsequent PCR.

In addition to the RNA molecule and the enzyme reverse transcriptase, a primer (e.g. random hexamer primer), desoxynucleosidetriphosphates and a salt pH balanced buffer are utilized to generate the mastermix. Further the mastermix contains an RNase inhibitor avoiding RNA degradation ([169]). The reverse transcription is performed in a thermocycler.

4.4.1 Materials

- Thermocycler personal (Eppendorf) or mastercycler personal (Biometra Personal Cycler)
- Reaction tubes, 1.5 ml (Biozym 710160)
- 0.2 ml PCR tubes (Biozym 71092; flat lid)
- Safe seal tips 100 µl (Biozym 770100)
- Safe seal tips 20 µl (Biozym 770050)
- Safe-Seal tips 10 µl (Biozym 770010)
- Gloves (e.g. Latex-gloves Peha-soft; Hartmann 942 161)
- Micropipettes

4.4.2 Reagents

- mRNA samples (integrity confirmed and concentration determined according to 0)
- Distilled water DNase/RNase free (Gibco 10977-035)
- High capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814) containing:
    - Reverse transcriptase (Applied Biosystems 4308228)
    - 10 x random hexamer primers (Applied Biosystems 4319979)
    - 100 mM desoxynucleosidtriphosphate (dNTP) mix (Applied Biosystems; 4367381)
    - 10 X reaction buffer (Applied Biosystems; 4319981)
    - Ribonuclease inhibitor (Applied Biosystems; N808-0119)

4.4.3 Reverse transcription (RT) of mRNA (first strand cDNA synthesis)

- Take 2 µg total RNA prepared according to (4.3)
- Add PCR water to a total volume of 10 µl
- For each sample prepare 10 µl reverse transcription mastermix (n = amount of samples; amount of mastermix = n + 1) according to table 2.

Table 2: Mastermix preparation

Component	Vol. for 1 sample in µl	Final Concentration in 20 µl RT reaction
10 x buffer	2	1 x
dNTPs (100 mM)	0.8	4 mM
10 x RT Random primer	2	1 x
MultiScribe Reverse Transcriptase (50 U/µl)	1	2.5 U
RNase inhibitor (20 U/µl)	1	1 U
PCR water	3.2	
Total volume	10	

- Centrifuge the samples briefly
- Add 10 µl mastermix to each sample (total volume 20 µl)
- Incubate the samples in the thermocycler applying the parameters indicated in table 3

Table 3: Temperature profile for RT of mRNA

Time [min]	Temp. [°C]
10	25
120	37
5	85
∞	4

- After the RT, add to every sample 20 µl PCR water to obtain a total volume of 40 µl containing 1000 ng cDNA (50 ng DNA in 1 µl)
- For subsequent PCR reactions 1 µl containing 50 ng cDNA was used
- The resulting cDNA is stored at -20 °C

4.5 Semiquantitative PCR

Following the RT of RNA into cDNA, the polymerase chain reaction (PCR) is applied to amplify the nucleotide sequence of interest. PCR reactions are used to determine expression of specific mRNA molecules in cell lines or tissues under investigation.

The mastermix, containing a Taq polymerase, a primer pair, dNTPs and a buffer is mixed with cDNA and PCR water. The PCR reaction is performed in the thermocycler.

The DNA polymerase is thermostable (Taq polymerase) and synthesizes the DNA ([167]), from nucleotides by using a single-stranded DNase template. The DNA primers bind during the **annealing** and mark the starting sequence for the enzyme. The synthesis of the sequence of interest begins at the opposite ends ([154]; [167]), reading the template from 3´ to 5´. After the **elongation** an elevation of the temperature (**denaturation step)** separates the new evolved double stranded DNA into its two single strands. New primers (sense and antisense primer) bind and the process recommences ([167]).

4.5.1 Materials

- Thermocycler personal (Eppendorf)
- Mastercycler personal (Biometra Personal Cycler)
- Reaction tubes, 1.5 ml (Biozym 710160)
- 0.2 ml PCR tubes (Biozym 71092; flat lid)
- PCR tubes with caps (Biozym 710900)
- Safe seal tips 100 µl (Biozym 770100)
- Safe seal tips 20 µl (Biozym 770050)
- Safe-Seal tips 10 µl (Biozym 770010)
- Gloves (e.g. Latex-gloves Peha-soft; Hartmann 942 161)
- Micropipettes

4.5.2 Reagents

- cDNA stored at-20 °C
- dNTPs mix (2 mM; Fermentas, R0241; 10 mM; Fermentas, R0192)
- β-actin sense primer (Eurofins MWG synthesis GmbH)
- β-actin antisense primer (Eurofins MWG synthesis GmbH)
- MT1 sense primer "Reppert", "Lanoix" (Eurofins MWG synthesis GmbH)
- MT1 antisense primer "Reppert", "Lanoix" (Eurofins MWG synthesis GmbH)

- Taq polymerase 1 U/µl (Fermentas, EP0404), supplied with:
    - 10 x Taq buffer with KCl
    - $MgCl_2$ (25 mM)
- Platinum® Taq Polymerase High Fidelity (Invitrogen, 11304-011, 5 U/µl,-20 °C)
- PCR water RNase/DNase free water (Gibco 10977-035)

4.5.3 Methods

cDNA amplification of β-actin by PCR

```
ATGGATGATGATATCGCCGCGCTCGTCGTCGACAACGGCTCCGGCATGTGCAAGGCCGGCTTCGCGGGCG
ACGATGCCCCCCGGGCCGTCTTCCCCTCCATCGTGGGGCGCCCCAGGCACCAGGGCGTGATGGTGGGCAT
GGGTCAGAAGGATTCCTATGTGGGCGACGAGGCCCAGAGCAAGAGAGGCATCCTCACCCTGAAGTACCCC
ATCGAGCACGGCATCGTCACCAACTGGGACGACATGGAGAAAATCTGGCACCACACCTTCTACAATGAGC
TGCGTGTGGCTCCCGAGGAGCACCCCGTGCTGCTGACCGAGGCCCCCCTGAACCCCAAGGCCAACCGCGA
GAAGATGACCCAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGTTGCTATCCAGGCTGTGCTA
TCCCTGTACGCCTCTGGCCGTACCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCCACACTGTGC
CCATCTACGAGGGGTATGCCCTCCCCCATGCCATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACTGA
CTACCTCATGAAGATCCTCACCGAGCGCGGCTACAGCTTCACCACCACGGCCGAGCGGGAAATCGTGCGT
GACATTAAGGAGAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAAGAGATGGCCACGGCTGCTTCCAGCT
CCTCCCTGGAGAAGAGCTACGAGCTGCCTGACGGCCAGGTCATCACCATTGGCAATGAGCGGTTCCGCTG
CCCTGAGGCACTCTTCCAGCCTTCCTTCCTGGGCATGGAGTCCTGTGGCATCCACGAAACTACCTTCAAC
TCCATCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTCTGGCGGCACCA
CCATGTACCCTGGCATTGCCGACAGGATGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGAT
CAAGATCATTGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCTGGCCTCGCTGTCC
ACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTATGACGAGTCCGGCCCCTCCATCGTCCACCGCAAAT
GCTTCTAG
```

Figure 8: cDNA sequence of human β-actin (CCDS5341.1). Positions of β-actin primers according to ([170]) are underlined and their corresponding 838 bp PCR product is shown in blue.

β-actin (ACTR) is ubiquitously expressed and serves as a positive control for the RT-reaction. Primers for β-actin cDNA amplification were selected according to ([170]) and result in an amplified cDNA fragment of 838 bp size (See Figure 8).

- Prepare the mastermix (n = amount of samples; n + 1 = amount of mastermix) according to table 4.

Table 4: β-actin mastermix preparation

Component	Vol. for 1 sample in µl	Final concentration in 25 µl PCR reaction
10 x buffer	2.5	1 x
MgCl$_2$ (25 mM)	1.5	1.5 mM
dNTPs (10 mM)	1	0.4 mM
β-actin sense primer (100 µM)	0.25	1 µM
β-actin antisense primer (100 µM)	0.25	1 µM
Taq Polymerase (1 U/µl)	1	1 U
RNase inhibitor (20 U/µl)	1	1 U
PCR water	5.0	
Total volume	12.5	

- Mix cDNA(prepared according to 4.4.3), PCR mastermix and H$_2$O to obtain a final volume of 25 µl
    - 12.5 µl mastermix
    - 1 µl cDNA (50ng cDNA/µl)
    - 11.5 µl PCR water
- Incubate samples in the thermocycler. For steps 2-4, 25 cycles are performed (according to table 5)
- Detection of the PCR products is performed according to (4.6.4)

Table 5: Amplification profile for β-actin

Step	Temperature	Time	Repetition
1	94 °C	2 min	1 x
2	94 °C	30 sec	1 x
3	61 °C	1 min	1 x
4	72 °C	1 min	1 x go to 2 for 25 x
5	72 °C	5 min	1 x
6	4 °C	-	∞

cDNA amplification of melatonin receptor 1 by PCR

MT1 cDNA amplification by PCR was performed using two different primer sets: Primer set 1 ("Reppert primers") was selected according to ([57]). PCR reactions performed with these primers result in amplification of a 286 bp cDNA fragment (See Figure 9).

Primer set 2 ("Lanoix primers") was selected according to ([21]). PCR reactions performed with these primers result in amplification of a 442 bp cDNA fragment (See Figure 10).

```
ATGCAGGGCAACGGCAGCGCGCTGCCCAACGCCTCCCAGCCCGTGCTCCGCGGGGACGGCGCGCGGCCCT
CGTGGCTGGCGTCCGCCCTGGCCTGCGTCCTCATCTTCACCATCGTGGTGGACATCCTGGGCAACC**TCCT
GGTCATCCTGTCGGTGTA**TCGGAACAAGAAGCTCAGGAACGCAGGAAACATCTTTGTGGTGAGCTTAGCG
GTGGCAGACCTGGTGGTGGCCATTTATCCGTACCCGTTGGTGCTGATGTCGATATTTAACAACGGGTGGA
ACCTGGGCTATCTGCACTGCCAAGTCAGTGGGTTCCTGATGGGCCTGAGCGTCATCGGCTCCATATTCAA
CATCACCGGCATCGCCATCAACCGCTACTGCTACATCTGCCACAGTCT**CAAGTACGACAAACTGTACAGC
AG**CAAGAACTCCCTCTGCTACGTGCTCCTCATATGGCTCCTGACGCTGGCGGCCGTCCTGCCCAACCTCC
GTGCAGGGACTCTCCAGTACGACCCGAGGATCTACTCGTGCACCTTCGCCCAGTCCGTCAGCTCCGCCTA
CACCATCGCCGTGGTGGTTTTCCACTTCCTCGTCCCCATGATCATAGTCATCTTCTGTTACCTGAGAATA
TGGATCCTGGTTCTCCAGGTCAGACAGAGGGTGAAACCTGACCGCAAACCCAAACTGAAACCACAGGACT
TCAGGAATTTTGTCACCATGTTTGTGGTTTTTGTCCTTTTTGCCATTTGCTGGGCTCCTCTGAACTTCAT
TGGCCTGGCCGTGGCCTCTGACCCCGCCAGCATGGTGCCTAGGATCCCAGAGTGGCTGTTTGTGGCCAGT
TACTACATGGCGTATTTCAACAGCTGCCTCAATGCCATTATATACGGGCTACTGAACCAAAATTTCAGGA
AGGAATACAGGAGAATTATAGTCTCGCTCTGTACAGCCAGGGTGTTCTTTGTGGACAGCTCTAACGACGT
GGCCGATAGGGTTAAATGGAAACCGTCTCCACTGATGACCAACAATAATGTAGTAAAGGTGGACTCCGTT
TAA
```

Figure 9: cDNA sequence of human Mel 1 (CCDS3848.1). Positions of the "Reppert" primers (according to [57]) are in red, underlined and their corresponding 286 bp PCR product is shown in red.

```
ATGCAGGGCAACGGCAGCGCGCTGCCCAACGCCTCCCAGCCCGTGCTCCGCGGGGACGGCGCGCGGCCCT
CGTGGCTGGCGTCCGCCCTGGCCTGCGTCCTCATCTTCACCATCGTGGTGGACATCCTGGGCAACCTCCT
GGTCATCCTGTCGGTGTATCGGAACAAGAAGCTCAGGAACGCAGGAAACATCTTTGTGGTGAGCTTAGCG
GTGGCAGACCTGGTGGTGGCCATTTATCCGTA**CCCGTTGGTGCTGATGTCGATATTTAACAACGGGTGGA
ACCTGGGCTATCTGCACTGCCAAGTCAGTGGGTTCCTGATGGGCCTGAGCGTCATCGGCTCCATATTCAA
CATCACCGGCATCGCCATCAACCGCTACTGCTACATCTGCCACAGTCTCAAGTACGACAAACTGTACAGC
AGCAAGAACTCCCTCTGCTACGTGCTCCTCATATGGCTCCTGACGCTGGCGGCCGTCCTGCCCAACCTCC
GTGCAGGGACTCTCCAGTACGACCCGAGGATCTACTCGTGCACCTTCGCCCAGTCCGTCAGCTCCGCCTA
CACCATCGCCGTGGTGGTTTTCCACTTCCTCGTCCCCATGATCATAGTCATCTTCTGTTACCTGAGAATA
TGGATCCTGGTTCTCCAGGTCAGACAGAGGGTGAAACCT**GACCGCAAACCCAAACTGAAACCACAGGACT
TCAGGAATTTTGTCACCATGTTTGTGGTTTTTGTCCTTTTTGCCATTTGCTGGGCTCCTCTGAACTTCAT
TGGCCTGGCCGTGGCCTCTGACCCCGCCAGCATGGTGCCTAGGATCCCAGAGTGGCTGTTTGTGGCCAGT
TACTACATGGCGTATTTCAACAGCTGCCTCAATGCCATTATATACGGGCTACTGAACCAAAATTTCAGGA
AGGAATACAGGAGAATTATAGTCTCGCTCTGTACAGCCAGGGTGTTCTTTGTGGACAGCTCTAACGACGT
GGCCGATAGGGTTAAATGGAAACCGTCTCCACTGATGACCAACAATAATGTAGTAAAGGTGGACTCCGTT
TAA
```

Figure 10: cDNA sequence of human MT1 (CCDS3848.1). Positions of the "Lanoix" primers (according to [21]) are underlined and red and their corresponding 442 bp PCR product is shown in red.

MT1 PCR with the hMT1 "Reppert"

To verify expression of hMT1 mRNA in cells, RT-PCR was performed. Primers were selected according to ([57]).

- Prepare the PCR mastermix (for n = number of samples prepare (n + 1) according to table 6

Table 6: MT1 ("Reppert") PCR mastermix preparation

Component	Vol. for 1 sample in µl	Final concentration in 25 µl RT reaction
10 x buffer	2.5	1 x
MgCl$_2$ (25 mM)	1.5	1.5 mM
dNTPs (2 mM)	2.5	0.2 mM
MT1 sense primer "Reppert" (100 µM)	0.25	1 µM
MT1 antisense primer "Reppert" (100 µM)	0.25	1 µM
Taq Polymerase (5 U/µl)	0.2	1 U
RNase inhibitor (20 U/µl)	1	1 U
PCR water	4.3	
Total volume	12.5	

- Mix cDNA, PCR mastermix and H$_2$O to obtain a final volume of 25 µl
  - 12.5 µl mastermix
  - 1 µl cDNA (50ng cDNA/µl)
  - 11.5 µl PCR water
- For amplification conditions in the thermo cycler see table 7. Steps 2-4 are continuously repeated for 40 times (= 40 cycles)

Table 7: Amplification profile for MT1 according to ([57])

Step	Temperature	Time	Repetition
1 Denaturation	94 ℃	3 min	1 x
2 Denaturation	94 ℃	30 sec	1 x
3 Annealing	65 ℃	30 sec	1 x
4 Elongation	72 ℃	30 sec	1 x go to 2 for 40 x
	4 ℃	∞	1 x

- Detection of the PCR products is performed according to (section 4.6.4)

MT1 PCR with hMT1 primers "Lanoix"

To verify expression of hMT1 mRNA in cells, RT-PCR was performed. Primers were selected according to ([21]).

- Prepare the PCR mastermix (for n = number of samples prepare (n + 1) according to table 8

Table 8: MT1 ("Lanoix") PCR mastermix preparation

Component	Vol. for 1 sample in µl	Final concentration in 20 µl RT reaction
10 x buffer	2.5	1 x
$MgCl_2$ (25 mM)	1	1 mM
dNTPs (2 mM)	2.5	0.2 mM
MT1 sense primer "Lanoix" (100 µM)	0.25	1 µM
MT1 antisense primer "Lanoix" (100 µM)	0.25	1 µM
Taq Polymerase (5 U/µl)	0.2	1 U
RNase inhibitor (20 U/µl)	1	1 U
PCR water	4.8	
Total volume	12.5	

- Mix cDNA, PCR mastermix and H_2O to obtain a final volume of 25 µl
  - 12.5 µl mastermix
  - 1 µl cDNA (50ng cDNA/µl)
  - 11.5 µl PCR water
- For amplification conditions in the thermo cycler see table 9. Steps 2-4 are continuously repeated for 40 times (= 40 cycles)

Table 9: Amplification profile for MT1 according to ([21])

Step	Temperature	Time	Repetition
1 Denaturation	94 ℃	8 min	1 x
2 Denaturation	94 ℃	30 sec	1 x
3 Annealing	**56 ℃**	45 sec	1 x
4 Elongation	72 ℃	1 min	1 x go to 2 for 40 x
5 Final elongation	68 ℃	7 min	1 x
	4 ℃	∞	1 x

- Detection of the PCR products is performed according to (section 4.6.4)

4.6 Native agarose gel electrophoresis

This technique is used to analyze the size of DNA molecules such as PCR molecules or to control intactness of RNA after isolation.

Following separation of intact RNA, two bands are visible under UV light, namely the 28 S and the 18 S rRNA band ([167]); the 28 S band should be nearly twice as intense as the 18 S band. If the RNA is degraded during RNA isolation, the bands will disappear and replaced by a "smear" of bands of low molecular weight.

Separation results from the movement of charged molecules in an electric field. The DNA and the RNA are negatively charged due to the phosphate groups forming the "backbone" of the molecules. Within an electric field they move towards the positive electrode (anode) in a matrix formed by the polysaccharide agarose. Pores in the agarose gel allow the separation of DNA/RNA molecules by size, as the larger molecules move slower than the small ones ([171]). The pore size of the gel depends on the percentage of agarose. A 1.5 % agarose gel has smaller pores then a 1 % gel and can therefore be used to separate smaller DNA/RNA molecules from each other ([167]).

For a consecutive visualization of RNA/DNA molecules in the gel the organic dye ethidium bromide (EtBr) is added to the agarose gel intercalating into DNA and RNA helices. When exposed to UV-light, it fluoresces with an orange contour. The fluorescence is almost 20-fold increased after binding to DNA/RNA ([167]).

4.6.1 Materials

- Microwave oven
- pH meter (827 pHLab, Metrohm)
- Transluminator (ChemiImager™ 4400)
- Stir plate (Framo® Gerätetechnik M20/1)
- Stir bar
- Electrophoresis chamber (PeqLab Biotechnology GmBH Model 41-1325)
- Power supply (PeqLab EV231)
- Gel casting tray
- 1000 ml beaker
- Graduated cylinder
- Erlenmeyer flask 250 ml

- 25 ml pipettes, autoclaved

- Gloves (e.g. Latex-gloves Peha-soft; Hartmann 942 161)

- Nitril gloves (TOP GLOVE sdn.bhd; 123 112 BLUE)

4.6.2 Reagents

- Agarose-1000 (Invitrogen 10975- 035)
- A.bid.
- Ethylendiamine tetraacetic acid (EDTA) sodium salt (Merck 1.08418.1000)
- Tris base (Merck 1.08382.1000)
- Glacial acetic acid (Merck 8.18755.2500)
- Bromphenolblue (Amresco 0449-25G)
- Xylene cyanol FF (Amresco 0819-20G)
- Glycerol (Merck 1.04094.0500)
- DNA marker Gene Ruler in Ladder 100 bp Plus DNA (Fermentas SM 0323; 0.1 µg/µl DNA)
- Ethidium bromide (Amresco 492-5g)

4.6.3 Buffers and media

0.5 M EDTA stock solution

For a 500 ml stock solution:
- Weigh out 93.05 g EDTA sodium salt (MW = 372.2)
- Dissolve in 400 ml A.bid.
- Adjust the pH with NaOH to a value of 8.0
- Adjust to a final volume of 500 ml
- Store at rt

EDTA will not go into solution completely until the pH is adjusted to about 8.0.

6x Loading Dye

- 0.25 % (w/v) Bromphenolblue
- 0.25 % (w/v) Xylene cyanol FF
- 30 % (w/v) glycerol in A.bid.
- Store in aliquots at-20 ℃

50 X stock solution of Tris acetate EDTA (TAE) buffer

For a 1 l stock solution:

- Weigh in 242 g Tris base (MW = 121.14)
- Dissolve in approximately 750 ml A.bid.
- Add carefully 57.1 ml glacial acetic acid
- Add 100 ml of 0.5 M EDTA (pH 8.0)
- Adjust the solution to a final volume of 1 l with A.bid.
- Store at rt

The pH of this buffer is not adjusted and it should be around 8.5.

1 X TAE buffer

- To 20 ml 50 X TAE stock solution
- Add 980 ml A.bid.
- Store at rt

4.6.3.1 Ethidium bromide stock solution

- Prepare a 10 mg/ml Etidium bromide solution in A. bid.
- Store at rt

4.6.4 Methods

Preparation of agarose gels

As ethidium bromide is a mutagen agent, it is necessary to wear nitril gloves when handling with the substance!

- To obtain a 1 % or a 1.5 % agarose gel, mix agarose with 1 X TAE buffer, according to table 10
- Boil briefly in the microwave oven
- Sway the mixture
- Boil briefely a second time
- Let the agarose solution cool down to 50-60 °C
- Add 12.5 µl ethidium bromide stock solution
- Pour the gel into the horizontal gel casting tray
- Place combs (for the gel slots)
- Allow the gel to solidify for 40 min
- Remove the combs and closings carefully

- If not immediately used, store gels at 4 °C, wrapp ed in saran wrap

Table 10: Agarose concentrations for gels

	1 % (w/v)	1.5 % (w/v)
Agarose	2.5 g	3.75 g
1 X TAE buffer	250 ml	250 ml

Sample preparation

- Mix PCR products (10 µl) or isolated RNA samples (10 µl) with 2 µl 6 x sample buffer

Native agarose gel electrophoresis

- Place gel on tray and insert in electrophoresis apparatus
- Cover the gel with 1 X TAE buffer
- Ensure that there are no bubbles in the slots
- Load the samples (12 µl/slot of a 28 comb)
- In one slot, load 3 µl of the DNA ladder
- Allow bands of interest to separate adequately at 5 V/cm (cm = distance between the electrodes), usually for 90 min

Gel evaluation via UV transilluminator

- The visualization and the photography should occur immediately after the separation via gel electrophoresis
- Gels are placed on an UV illuminator and the fluorescing RNA bands are photographed
- Store pictures as .tif files and process by Adobe Photoshop

4.7 Preparation of cell lysates, Bradford assay, SDS-PAGE and western blotting

4.7.1 Materials

- Microcentrifuge (Eppendorf 5415R)
- Spectrophotometer (Hitachi U-2800A spectrophotometer)
- Heating block (Eppendorf; Thermomixer comfort)
- pH meter (827 pHLab, Metrohm)
- Gel electrophoresis apparatus (LKB 2050 Midget)
- Power supply (LKB 2297 Macrodrive 5)
- Blotting apparatus (peqLab Biotechnology GmBH; Semi-Dry Electroblotter)
- Scanner (Hp scanjet 5470c)
- Film developing machine (Agfa GP 1000)
- Film-casette (DuPont)
- Wet chamber (box)
- Erlenmeyer flask (250 ml, 100ml)
- Beaker (200 ml, 100 ml)
- Cell scrapers (Sterilin 9010-0320)
- Pipette boy (Peqlab)
- Micropipette
- Tubes 1.5 ml (Biozym 710310)
- 230 mm glass pasteur pipettes, sterile (VWR 612-1702)
- Sterile tips
- Loading tips (Peylab 81-28480)
- 1000 µl tips (Biozym 721000)
- 200 µl tips (Biozym 770280)
- Extra thick blot paper (Bio-Rad 1703966)
- Polyvinylidene fluoride membrane (Pall Corporation 75696G)
- Amersham Hyperfilm ECL (GE Healtcare 28-9068-36)
- Saran wrap (e.g. 6-l-Gefrierbeutel Profissimo)
- Filter paper (Whatman # 1)
- Gloves (e.g. Latex-gloves Peha-soft; Hartmann 942 161)
- Ice

4.7.2 Reagents:

- 2-Mercaptoethanol (Sigma M-7154)
- A. bid.
- Acetic acid (Merck 1.18755.2500)
- Acrylamide Bis solution 40 %, 19 : 1 (Biorad 161-0144) for the stacking gel
- Acrylamide Bis solution 40 %, 37.5 : 1 (Biorad 161-0148) for the separation gel
- Ammonium perulfate (AMPS; Sigma A-3678)
- Bovine serum albumine (BSA; SAFC A8806)
- Coomassie brilliant blue G250 (LKB 1840-102)
- Dry milk powder (e.g. Maresi)
- EDTA (Merck 1.08418.1000)
- Ethanol (Merck 1.00983.1000)
- Glycerol; about 87 % (Merck 1.04094.0500)
- Glycine (Merck 1.04169.1000)
- HCl, fuming 37 % (Merck 1.00314.1000)
- HEPES (Sigma H3375-500G)
- Human IgG for standard curve (Endobulin S/D Baxter)
- Kaleidoscope molecular weight marker; Page ruler, prestained protein ladder (Fermentas SM 0671)
- Liquid nitrogen
- Methanol (Fisher Scientific M/4000/17)
- $MgCl_2$ (Merck 1.05833.1000)
- n-Butanol (1-Butanol; Merck 1990)
- Phosphoric acid, 85 % (Merck 573.1000)
- Ponceau S, practical grade (Sigma P-3504)
- Primary and HRP-conjugated secondary antibodies
- Sodium dodecylsulfate (SDS; Sigma L-3771)
- Super Signal® West Pico Chemiluminescent (Thermo Scientific 34080)
- Tetramethylethylendiamin (TEMED; Biorad 161-0801)
- Tris (Merck 1.08382.1000)
- Triton X-100 (TX-100; Merck 11869-1000)
- Tween 20 for electrophoresis (Sigma P-5927)

- Protease inhibitor cocktail (Sigma-P8340), stored at-20 ℃ in aliquots of 50 µl, final dilution: 1 : 100

  It contains:

  - AEBSF-[4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride] (inhibits serine proteases)
  - Aprotinin (inhibits serine proteases)
  - Bestatin hydrochloride (inhibits aminopeptidases)
  - E-64-[N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide] (inhibits cysteine proteases)
  - Leupeptin hemisulfate salt (inhibits serine and cysteine proteases)

4.7.3 Buffers and media

PBS (4.1.3)
Cell lysis buffer

- 0.2 ml TX-100 were added to 200 ml PBS
- Final concentration 0.1 % (v/v)
- Store at rt

Bradford reagent

- Dissolve 100 mg Coomassie Blue G-250 in 50 ml ethanol (95 % (v/v))
- Stir at rt
- Add 100 ml (85 % (w/v)) phosphoric acid and stir o/n at rt
- Adjust with A.bid. to a final volume of 1 liter after the dye has completely dissolved
- Stir at rt
- Filter through Whatman # 1 paper
- Store at rt, protected from light (brown flask)

If the reagent changes its usual light brown colour and turns blue, the reagent does not work anymore and should be discarded.

IgG standard solution

- Dissolve hIgG in A.bid. at a concentration of 1 mg/ml
- Aliquot (50 µl) and store at-20 ℃

10 % AMPS solution

- Weigh in 1 g AMPS

- Dissolve in 10 ml A.bid.
- Aliquote 1 ml
- Store at-20 ℃

Washing buffer PBS containing 0.1 % v/v Tween[®] 20

- 1 ml Tween[®] 20
- Add 999 ml PBS
- Store at rt

10 % (w/v) SDS solution

- Weigh in 10 g SDS
- Dissolve in 100 ml A.bid.
- Store at rt

3 M Tris-HCl, pH 8.8 (500 ml)

- Weigh in 181.7 g Tris
- Dissolve in about 450 ml
- Adjust pH 8.8 with HCl
- Adjust with A.bid. to a final volume of 500 ml
- Store at rt

0.5 M Tris-HCl, pH 6.8 (100 ml)

- Weigh in 6.1 g Tris
- Dissolve it in about 80 ml A.bid.
- Adjust pH 6.8 with HCl
- Adjust to a final volume of 100 ml
- Store at rt

SDS-PAGE sample buffer (4 x stock solution; [67])

- Dissolve 0.378g Tris (62.6 mM) in 30 ml A.bid.
- Adjust pH to 6.8 with 0.1 N HCl.
- Add and dissolve the 2.5 g SDS and the 5.74 ml Glycerol
- Adjust volume to 50 ml with additional A.bid.
- Control again the pH-value to make sure that it is 6.8
- Aliquote the 50 ml buffer (1 ml/1.5 ml tube)
- Store the buffer at -20 ℃

Sample buffer (1 X)

- Mix 1 vol. 4 x stock solution and 3 vol. A.bid.
- Final concentrations: Tris 62.5 mM; Glycerol 6.5 %; Sodium dodecylsulfate (SDS) 2 % (w/v); 2-mercaptoethanol (5 %)

Running buffer (10 X stock solution)

- Weigh in
    - 30 g Tris (0.25 M)
    - 144 g Glycine (39 mM)
    - 10 g SDS (1 % w/v)
- Dissolve in A.bid.
- Bring to a final volume of 1000 ml with A.bid.
- Store at rt

Running buffer (1 X)

- Mix 1 vol. 10 X stock solution and 9 vol. A.bid.
- Store at rt
- Final concentrations
    - Tris 25 mM
    - Glycine 192 mM
    - SDS 0.1 % (w/v)

Transfer buffer

- Weigh in
    - 5.8 g Tris (48 mM)
    - 2.93 Glycin (39 mM)
    - 0.375 g SDS (0.0375 % (w/v))
- Add 200 ml MeOH (20 % (v/v))
- Dissolve in 600 ml
- Bring to a final volume of 1000 ml with A.bid.

Ponceau red staining buffer

- Weigh in 0.5 g ponceau red (0.1 % (w/v))
- Add 25 ml (5 % (v/v)) acetic acid
- Dissolve in A.bid.
- Bring to a final volume of 500 ml with A.bid.

Ponceau red destaining solution

- 5 ml acetic acid (1 % (v/v))
- Dissolve in A.bid.
- Bring to a final volume of 500 ml with A.bid.

Blocking buffer (= Blotto)

- Weigh in 25 g fat-free milk powder (5 % (w/v))
- Dissolve
- Bring to a final volume of 500 ml with 0.1 % (v/v) PBS/Tween® 20

Coomassie blue staining solution

- Weigh in 1.25 g Coomassie brilliant blue (0.25 % (w/v))
- Dissolve
- Bring to a final volume of 500 ml of a solution containing: methanol : A.bid. : acetic acid (5 : 5 : 1)

Coomassie blue destaining solution

- 25 ml methanol (5 % (v/v))
- 50 ml acetic acid (10 % (v/v))
- Dissolve and bring to a final volume of 500 ml with A.bid.

Restore™ Western Blot Stripping buffer

(Thermo Scientific; 21059)

4.7.4 Methods:

Preparation of PBS-TX-100 proteine lysates

Work on ice!

- Cells should be grown ~ 80-90 % confluent
- Transfer the culture flasks with cells from the cell culture laboratory to the bench
- Wash the cells with PBS (5 ml/25 cm^2; 15 ml/75 cm^2)
- Aspirate the PBS
- Add PBS (0.5 ml/ 25 cm^2 culture flask; 1 ml/75 cm^2 culture flask)
- Harvest cells with a cell scraper
- Transfer cells in a microcentrifuge tube
- Pellet the cells by centrifugation (200 x g, 4 °C)

- Discard the supernatant
- Add ice-cold lysis buffer which contains a protease-inhibitor cocktail (0.2 ml/25 cm^2 culture flask; 0.6 ml/75 cm^2 culture flask)
- Vortex at 4 °C for 30 min
- Centrifuge in the microcentrifuge at 12000 x g, 5 min, 4 °C to remove the insoluble material
- Transfer the supernatant in a fresh tube
- Discard the pellet
- Aliquote lysates → 100 µl
- Store the lysates at - 20 °C or at - 80 °C for lon g time storage

The proteins should not be thawed and frozen repetitively, because protein gel degrade!

Bradford Protein Assay

- Pipette IgG standard solution (0, 2, 4, 8, 10, 12 µl) in duplicate into tubes
- Pipette aliquots of samples of interest in duplicate into centrifuge tubes (sample volumes need to be determined empirically: try 2-12 µl of undiluted (cell lysates) or diluted (tissue lysates) samples)
- Add 1 ml Bradford reagent to each tube, vortex
- Incubate at rt for 10 min (the complex dye-protein is stable for ∼ 1 h)
- Measure the absorbance of the standard curve samples at 595 nm in a spectrophotometer (turn on 15 min before use) and allow the instrument to calculate a standard curve
- Measure of all other samples
- Calculate protein concentration in samples using the standard curve

SDS-PAGE (<u>s</u>odium - <u>d</u>odecylsulfate - <u>p</u>olyacrylamide - <u>g</u>el - <u>e</u>lectrophoresis; [172])

SDS-PAGE allows protein separation in an electric field based on their size ([169]). The separated proteins move towards the anode. Tertiary and quarternary structures of the proteins are destroyed by boiling in the presence of SDS and 2-Mercaptoethanol reducing the disulfide bonds and introducing a negative charge.

The pore size of the gel can be changed by augmentation or reduction of the acrylamide concentration. The higher the acrylamide concentration is, the smaller is the pore size ([173]).

Preparing the separation gel for the SDS PAGE

All materials used like plates, combs and spacers should be clean. Wash the materials with detergent, water and thereafter with EtOH; allow them to dry.

For one gel, assemble a casting stand made of aluminiumhydroxid plates, spacers (0.75 mm) and glass plates and fix.

- Mix 40 % acrylamide bis solution for separation gels, 3 M Tris HCl, 10 % SDS and A.bid. according to table 11
- For inducing the gel polymerisation, add AMPS (ml) and TEMED (in µl), according to table 11
- Mix all ingredients
- Immediately pour the gels; leave enough free space for the stacking gel and combs
- Overlay each gel with n-Butanol (1-Butanol) saturated with A.bid. (upper layer) to obtain vertical surface of the separation gel
- Allow the gel to polymerize for at least 1 h at rt
- If the gels are not needed immediately, it is possible to wrap them one by one in saran wrap, to place them in a wet-chamber and to store them for up to 2 weeks at 4 ℃

Table 11: Recipes for the preparation of SDS-PAGE separation gel. The recipe´s volumes for the separation gels are in ml, except for TEMED!

%	Acrylamide Bis solution (40%) 37.5 : 1	Tris HCl (3M, pH 8.8)	SDS 10%	A. bid.	AMPS (10 %)	TEMED in µl
6	5.8	5	0.4	28.4	0.4	40
7.5	9.4	5	0.4	33.4	0.4	40
10	**12.5**	**5**	**0.4**	**30.3**	**0.4**	**40**
12	15	5	0.4	27.8	0.4	40
15	16.9	5	0.4	21.6	0.4	40

Preparation of the stacking gel for the SDS-PAGE

- Fix single gels in the gel electrophoresis apparatus
- Before pouring the stacking gel make sure that no fluid is left on the surface of the separation gel. Remove the fluid with a filter paper
- For the stacking gel, mix Acrylamide Bis solution (40 %) 19 : 1, Tris HCl (0.5 M, pH 6.8) SDS (10 %) and A.bid. according to table 12
- Add AMPS and TEMED, according to table 12
- Mix and immediately pour gels
- Overlay the separation gel with the stacking gel solution (~ 2 ml/gel)
- Insert 1 comb/stacking gel (10 wells, 0.75 mm). Make sure not to include air bubbles
- Allow to polymerize for at least 30 min at rt

Table 12: Recipe for the SDS-PAGE stacking gel (for 4 gels)

%	Acrylamide Bis solution (40%) 19 : 1	Tris HCl (0.5M, pH 6.8)	SDS (10%)	A.bid.	AMPS (10%)	TEMED
4	1.5 ml	1.9 ml	150 µl	11.6 ml	150 µl	20 µl

- After completed polymerization, remove the combs carefully
- Remove carefully any remaining fluid from the slots with the water jet pump
- Overlay the gel with 1 x running buffer

Preparation of the samples

- Determine protein concentration of samples according to (0)
- Calculate the volume needed to load a certain protein amount/slot
- Mix sample with 4 x sample buffer and water to obtain a final volume of 20 µl
- Heat the samples for 5 min at 95 ℃ in the heating block

SDS-PAGE

- In the meantime, add 1 X running buffer to the upper buffer reservoir
- Load the samples and the marker with the loading tips into the gel slots (Using 0.75 mm spacers; 20 µl of the sample/1 slot and 5 µl of the marker were applied)
- Empty slots should be filled with 1 x SDS-PAGE sample buffer
- Add 1 X running buffer to the lower buffer reservoir

- Close the lid of the electrophoresis apparatus
- Run the gel at 150 V
- Stop the electrophoresis when the Bromphenol blue in the sample buffer has reached the bottom of the gel

Western blotting

After separation by SDS-PAGE and electrophoretic transferation of the proteins to a PVDF-membrane, the antigens of interest can be detected by specific mono-or polyclonal antibodies through immunoblotting ([174]).

The immunoblotting starts with the blocking of unspecific binding sites of the proteins, bound to the surface of the PVDF-membrane by immersing the membrane in blocking buffer and is followed by the incubation with the 1st antibody and a washing step. The 2nd antibody is an enzyme conjugated antibody, which binds onto the 1st antibody-antigene complex. The enzyme of the 2nd antibody can be HRP (horse radish peroxidase) or AP (alkaline phosphatase) whereas the antibody is an anti-IgG. For a subsequent incubation with other antibodies or with the same antibodies in other concentrations the membranes are stripped and reprobed ([174]).

Electroblotting (Semi-dry blotting)

- During the protein separation via gel electrophoresis, prepare for each gel one piece of PVDF membrane, with the same size as the gel, and two pieces of extra-thick gel blot paper
    - Pre-wet the blotting paper in the transfer buffer
    - The PVDF membrane is very hydrophobic, therefore pre-wet it first for 1 min in methanol. Afterwards put it in A.bid. and finally in the transfer buffer for about 10 min
- After stopping the gel electrophoresis, remove the stacking gel and put the seperation gels in transfer buffer
- Assemble the transfer stack by starting with the anode, followed by pre-wetted extra thick blot paper-PVDF membrane-separation gel-pre-wetted extra thick blot paper-cathode

    Make sure that the stack is not too wet and that there are no air bubbles included
- For the electric transfer of the proteins use the following conditions:
    - 90 min at 3 mA/cm^2

        One gel has a surface of ~ 8 x 5 cm = 40 cm^2 x 3 =   120 mA (for one gel)

        240 mA (for two gels)

Detection of the separated proteins (Coomassie blue staining)

Following transfer, the proteins remaining in the gel can be detected by Coomassie staining. In combination with Ponceau staining of the membran this gives an idea wether transfer conditions were sufficient. The Coomassie blue staining solution contains methanol and acetic acid needed for the precipitation of the separated proteins.

The dye interacts with proteins in an unspecifical manner. It is hypothesized that it works as an anion in the staining solution and binds onto the NH_3^+ groups of the amino acids, forming separated proteins. After destaining the proteins show up as blue bands on a clear background due to the fact that polyacrylamide gels do not interact with Coomassie brilliant blue ([175]).

- For the staining, incubate the gel in Coomassie blue staining solution for 30 min at rt (shaking)
- Afterwards destain the gel with Coomassie blue destaining solution for several hours (shaking)
- Change the buffer several times and add a sponge to increase destaining
- Dry gel on a piece of filterpaper for 2 h at 80 ℃ under vacuum
- Scan the dried gel and save it as .tif-file

Reversible staining of proteins on the membrane by Ponceau S

The successful transfer to the membrane can be controlled via staining with Ponceau S.

A destaining after the control is possible, because this technique does not fix the proteins to the transfer membrane and permits therefore the following western blotting ([174]).

- Place the PVDF membrane for 5 min in the Ponceau red staining solution (shaking)
- Destain the background of the membrane (reduce unspecific staining) by incubating the membrane for 5 min in Ponceau red distaining solution (shaking)
- Wrap the PVDF membranes in e.g. saran wrap
- Scan the membrane as .tif-file
- For the complete destaining of the proteins, wash the blotting membranes for about 10 min with PBS

Blocking of unspecific binding sites on the PVDF membrane

- To prevent unspecific binding of the antibodies to the PVDF membrane, incubate the blots in blocking buffer for 1 h at rt or over night at 4 ℃

Incubation with the antibodies

- Dilute antibodies in blocking buffer at the concentrations indicated in the results
- Seal blots with 2 ml antibody/blot in a plastic bag to reduce the amount of antibody needed
- Incubate the blots with the first antibody for 1 h at rt or over night at 4 °C (shaking)
- Wash the blots with washing buffer
- Change the buffer after each of the three washing steps. Every washing step should last for about 15 min
- Incubate the blots with the secondary, HRP conjugated antibody for 1 h at rt or over night at 4 °C (shaking). Dilute secondary anti body in blotting buffer to concentrations indicated in results
- Wash the blots again three times for 15 min with washing buffer. Change the buffer after each washing step
- To evaluate specificity of binding, blocking peptids were applied in some cases

Preincubation of the primary antibody with blocking peptide to evaluate the specifity of the binding

In this work the blocking peptide corresponded to a part of the sequence of the melatonin receptor MT1.

- Prepare two 1.5 ml tubes with 10 µl primary antibody (SC-13179), i.e. 2 µg
- Add an 5 x excess (by wheight) of blocking peptide (SC 13179 P) to tube A, i.e. 10 µg in 50 µl
- Add the same volume of PBS to tube (50 µl) B
- Add PBS to both tubes to a final volume of 500 µl
- Incubate the tubes for 2 h at rt (shaking) for pre-absorption of the antibody in tube A
- Add 1.5 ml blocking buffer to each tube (final dilution of the antibody 1 : 200)
- Add the two antibody solutions to 2 blots both prepared in the same manner
- Proceed as described in section "Incubation with the antibodies"

Detection of the HRP activity via chemoluminescence

- Binding of 1st and respective of the 2nd antibody is demonstrated via the HRP-activity of the latter by chemoluminescence (CL)
- Mix the two solutions of the detection reagent according to the instructions of the manufacturer. Use 2 ml for each blot of the mix

- Drain the wash buffer from the washed membranes
- Incubate the membranes for 5 min at rt with the mixture of the detection reagent (turn off the lights during this incubation step)
- Hold the blots against a paper towel to drain off excess reagent
- Wrap the blots in a fresh piece of saran wrap and try to avoid air bubbles
- Mark the edges of the membrane on the saran wrap
- Place the blots in an x-ray film cassette and fix the blots with a tape. The protein side is up
- Move to the darkroom and work from now on in the dark
- Adjust the size of a sheet of Hyperfilm ECL and cut the upper, right corner of the sheet
- Place it on the top of the membrane
- Close the cassette
- Expose for a certain time. Usually the first exposition time is about 20 sec, to see how strong the signal is. For further expositions time can vary from a few seconds to several hours
- Work fast because the signal gets lower in time
- Scan the developed films
- Store the data as .tif-files

Stripping

- Wash the membranes for 15 min with washing buffer (4.7.3)
- Incubate the blots with the stripping buffer for 15 min (10 ml for 2 membranes)
- Wash the membranes for 15 min with washing buffer (4.7.3)

5 Results

5.1 Optimization of a lipid-based transfection protocol for BeWo cells and establishment of RT-PCR protocols as tools for overexpression of MT1 and for detection of MT1 mRNA, respectively

5.1.1 Optimization of a lipid-based transfection protocol for BeWo cells

Before, we have already successfully used the BeWo cell line to study functions of placental proteins such as the human neonatal Fc receptor, hFcRn, not only based on the endogenous expression but also following induction of protein expression by stable transfection of the cells with the hFcRn cDNA using the calcium phosphate transfection protocol ([106]; [159]; [107]; [108]; [100]). Similarly, and in order to generate BeWo cells with elevated expression of MT1, the cDNA for human MT1 linked N-terminally to a triple HA-tag and cloned into the mammalian expression vector pcDNA3, was recently used to transfect BeWo cells with the calcium phosphate transfection protocol ([166]). Following antibiotic selection of stably expressing clones, we were able to confirm several-fold elevation of expression of MT1 mRNA in various clones by RT-PCR and real-time PCR, however, we could not demonstrate any significant elevation of MT1 expression at the protein level, neither with an anti-human MT1 antibody (Santa Cruz sc-13179) nor an anti-HA antibody (GeneTex GTX211424) used in that study. We speculated that the antibodies used in that study were not able to detect the HA-tagged fully processed MT1 protein, or alternatively, HA-tagged MT1 was not expressed at the protein level.

To be more flexible in detection of MT1 protein, we now decided to re-transfect BeWo cells with the cDNA for triple HA-tagged human MT1 (3xHA-MT1) as well as human MT1 cDNA either tagged with an N-terminal flag peptide (Flag-MT1) or a C-terminal green fluorescence protein, GFP (MT1 GFP; [165]; [164]; [60]). In addition to BeWo cells that are polarized epithelial cells of placental origin, we determined to also transfect MDCK II cells (Madine-Darby canine kidney cells II ([160]), that form epithelial monolayers ([161]) and present a well-accepted model to study protein traffic (especially receptor traffic) in epithelial cells ([162]). The expression of MT1 mRNA/protein in MDCK II cells is unknown, but from the non-detected melatonin binding ([163]) it must be concluded that these cells do not express MT1 or any other high-affinity melatonin receptor, at least at the protein level.

To circumvent some of the disadvantages of the calcium phosphate transfection protocol (e.g. low reproducibility), we decided to establish a lipid-based transfection protocol using FuGENE® HD transfection reagent (Roche), that is compatible not only with both transient and stable transfection, but also many cell types and exhibits low toxicity. Optimization of the transfection protocol required (1) determination of an optimal cell density, i.e. 80-90% confluency and (2) evaluation of the optimal transfection reagent to DNA ratio. The optimization procedure was performed by transfection of the plasmid pGFP-N3 (Clontech). Successful (transient) transfection of this vector results in cytoplasmatic expression of the green fluorescent protein that is easily detected by direct fluorescence microscopy.

Establishment of optimal seeding numbers of BeWo and MDCK II cells for transfection

Following determination of cell counts according to (4.1.6), various numbers BeWo as well as MDCK II cells were seeded at various concentrations in 24 well plates (4.1.6). 24 hours after seeding, confluency of the cells was estimated by phase contrast microscopi evaluation using the Axio Observer Z1, an inverted microscope that allowed analysis of living cells in the cell culture plate without prior fixation (4.2). Examples for the grade of confluency corresponding to the respective seeding numbers are shown in Figure 11 and Figure 12. Based on the evaluation of several fields of view/seeding concentration and cell line, a seeding number of 1.44×10^5 BeWo cells/2cm^2 and 0.72×10^5 MDCK II cells/2cm^2 (i.e. 1 well of a 24 well plate) was established as optimal cell number to obtain 80-90% confluency after 24 hours.
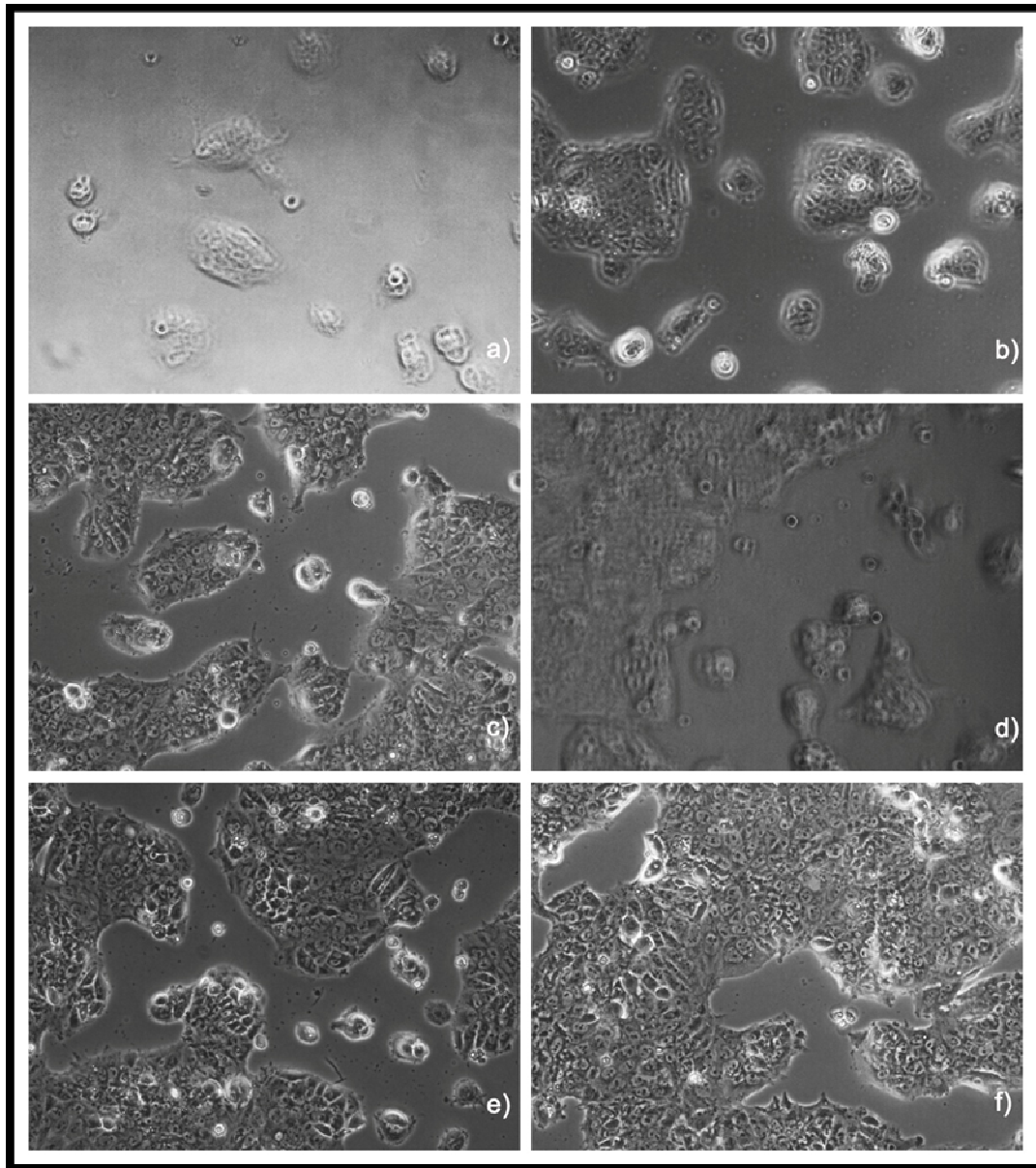
Figure 11: Confluency of BeWo cells 24 hours after seeding of (a) 0.24 x 10^5, (b) 0.48 x 10^5; (c) 0.72 x10^5; (d) 0.96 x 10^5; (e) 1.2 x 10^5; (f) 1.44 x 10^5 cells/well of a 24-well plate. Pase contrast microscopy was done on unfixed cells with a 10x objective. The optimum cell number for MDCK II cells to reach 80-90% confluency as estimated from several fields of view/well was 1.44 x 10^5 (f).
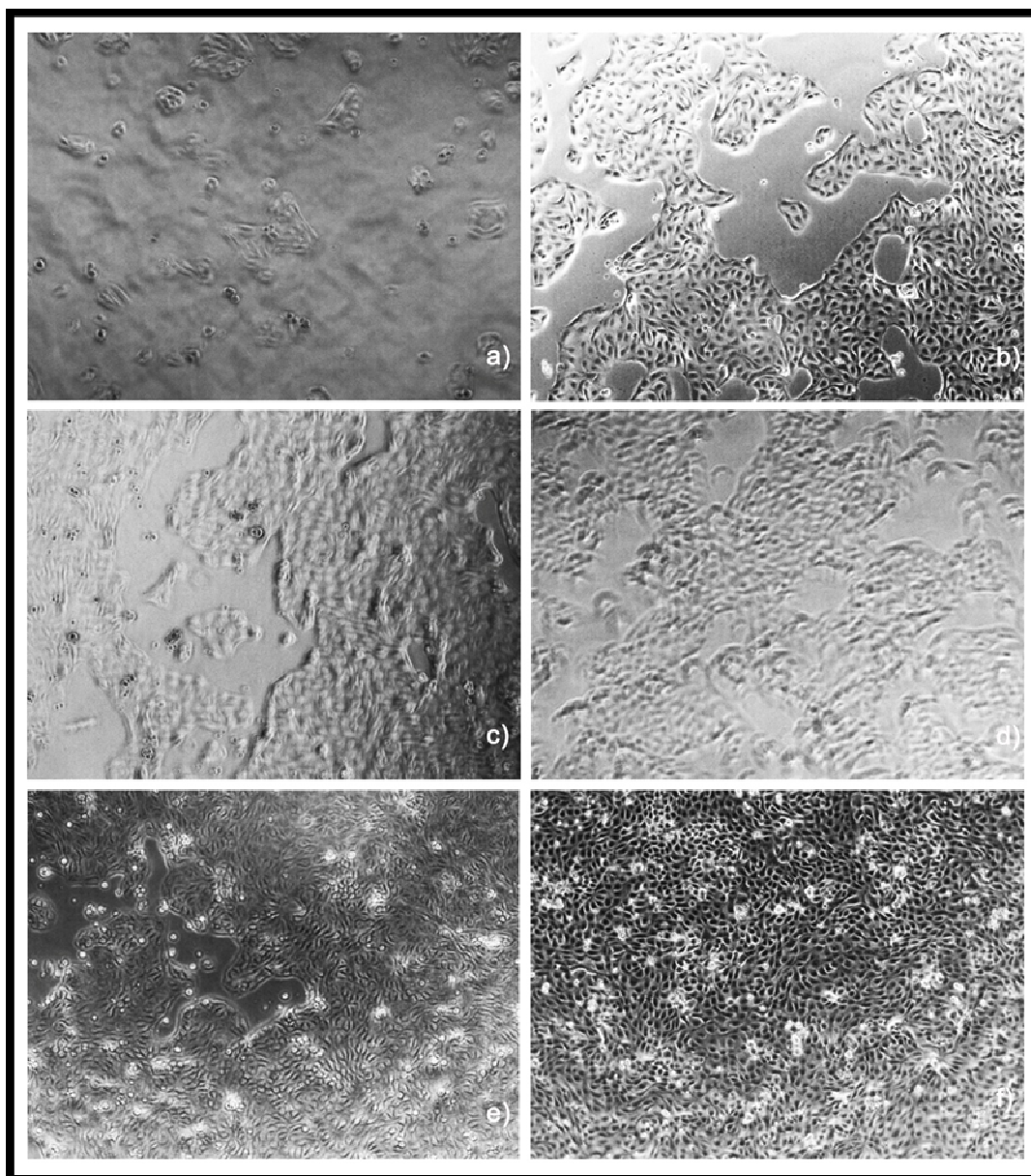
Figure 12: Confluency of MDCK II cells 24 hours after seeding of (a) 0.24 x 10^5, (b) 0.48 x 10^5; (c) 0.72 x10^5; (d) 0.96 x 10^5; (e) 1.2 x 10^5; (f) 1.44 x 10^5 cells/well of a 24-well plate. Pase contrast microscopy was done on unfixed cells with a 10x objective. The optimum cell number for MDCK II cells to reach 80-90% confluency as estimated from several fields of view/well was 0.72 x 10^5 cells (c).

Establishment of an optimal ratio of transfection reagent to DNA

BeWo cells as well as MDCK II cells were seeded in the optimal concentration in 24 well plates (4.1.6). 24 hours later, when the cells had reached a confluency of 80-90%, cells were transfected with the plasmid pGFP-N3 as detailed in the materials and method section (4.1.6). DNA purity of the plasmid was estimated by determination of the 260 nm/280 nm ratio and was found to be 1.87, thereby exceeding the required value of 1.8. For both cell lines, 2 µg of plasmid were mixed with increasing amounts of FuGENE$^®$ HD transfection reagent (5-12 µl). 24 hours after the transfection, living cells were examined by direct fluorescence microscopy using the Axio Observer Z1 according to 2.2. Successfully transfected cells that expressed the GFP protein were identified by their bright cytoplasmic green fluorescence. Results are exemplified by 1 field of view /cell line and condition in Figure 13 and 14. For both cell lines, BeWo (c24) and MDCK II, a ratio FuGENE$^®$ HD : plasmid DNA of 9 : 2 was selected as optimum composition (Figure 7 and 8 c), resulting in a transfection efficiency of about 10-20%. Below that ratio (Figure 7 and 8 b and not shown for 5-7 µl of FuGENE), significant lower amounts of transfected cells were observed. Above that ratio, no significant increase in the amount of transfected cells was observed. Therefore, the ratio FuGENE$^®$ HD : plasmid DNA of 9 : 2 was used for all subsequent transfections of the cells to save transfection reagent. Although these efficiencies appear not that high, similar transfection efficiencies have been reported by others for BeWo as well as MDCK II cells using the FuGENE$^®$ HD reagent (e.g. [176]; **www.biontex.com/con_4_6_4/cms/upload/pdf/Deen_MP_en.pdf**)
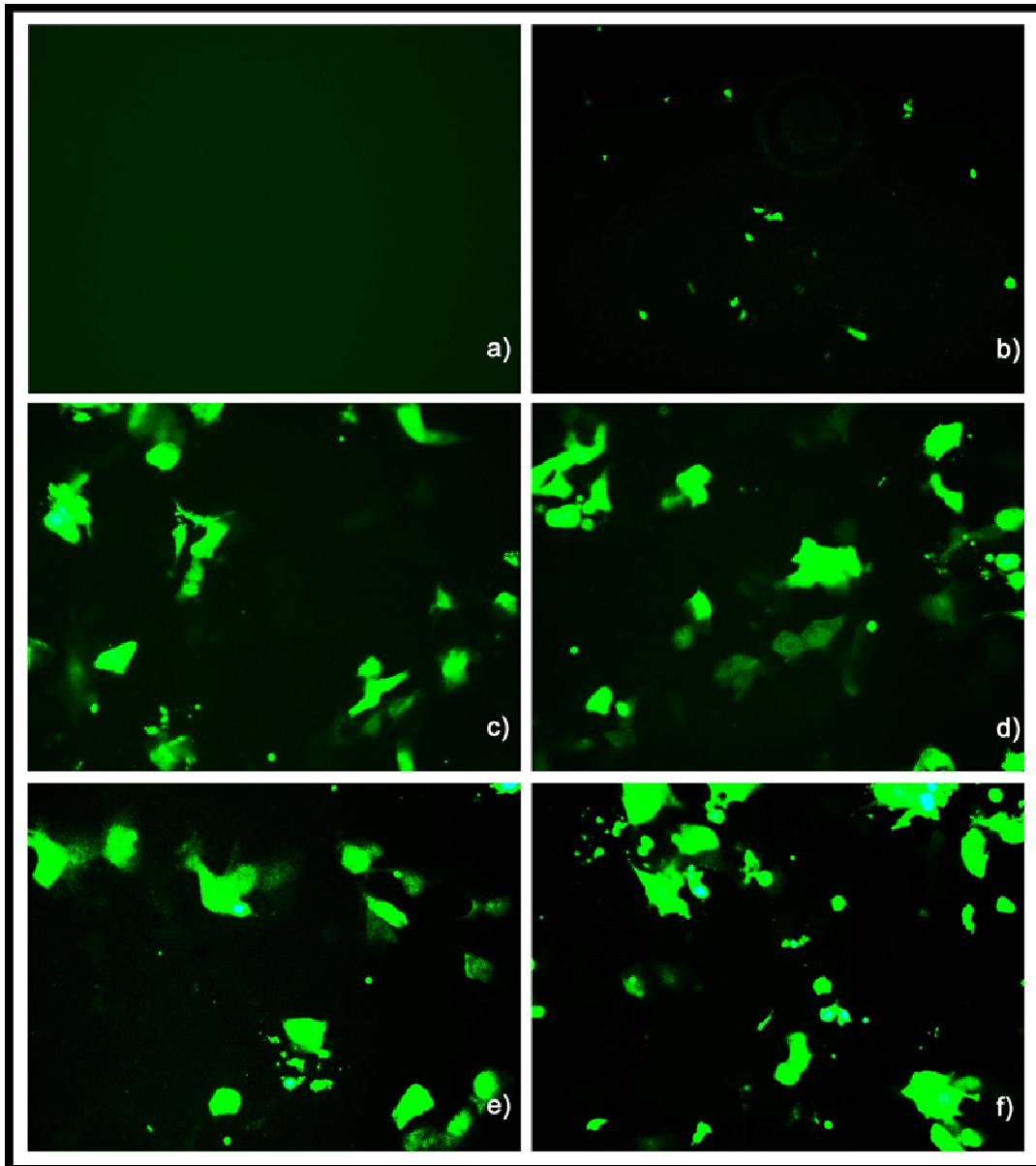
Figure 13: Transfection efficiency of BeWo cells following transient transfection with the plasmid in reaction to pGFP-N3 and increasing volumes of transfection Reagent (FuGENE® HD). BeWo cells were cultured for 24 hours to a confluency of 80-90%. Cells were transfected either without DNA (a) or 2 µg DNA (b-f) in the presence of (b) 8, (c) 9, (d) 10, (e) 11 or (f) 12 µl of FuGENE® HD. The pictures (a) and (b) were taken with a 10x objective, (c-f) with a 32x objective using an inverted microscope and an excitation/emission filter set appropriate to detect of the GFP-fluorescence. A ratio of FuGENE® HD : DNA of 9 : 2 (c) was selected for further transfections
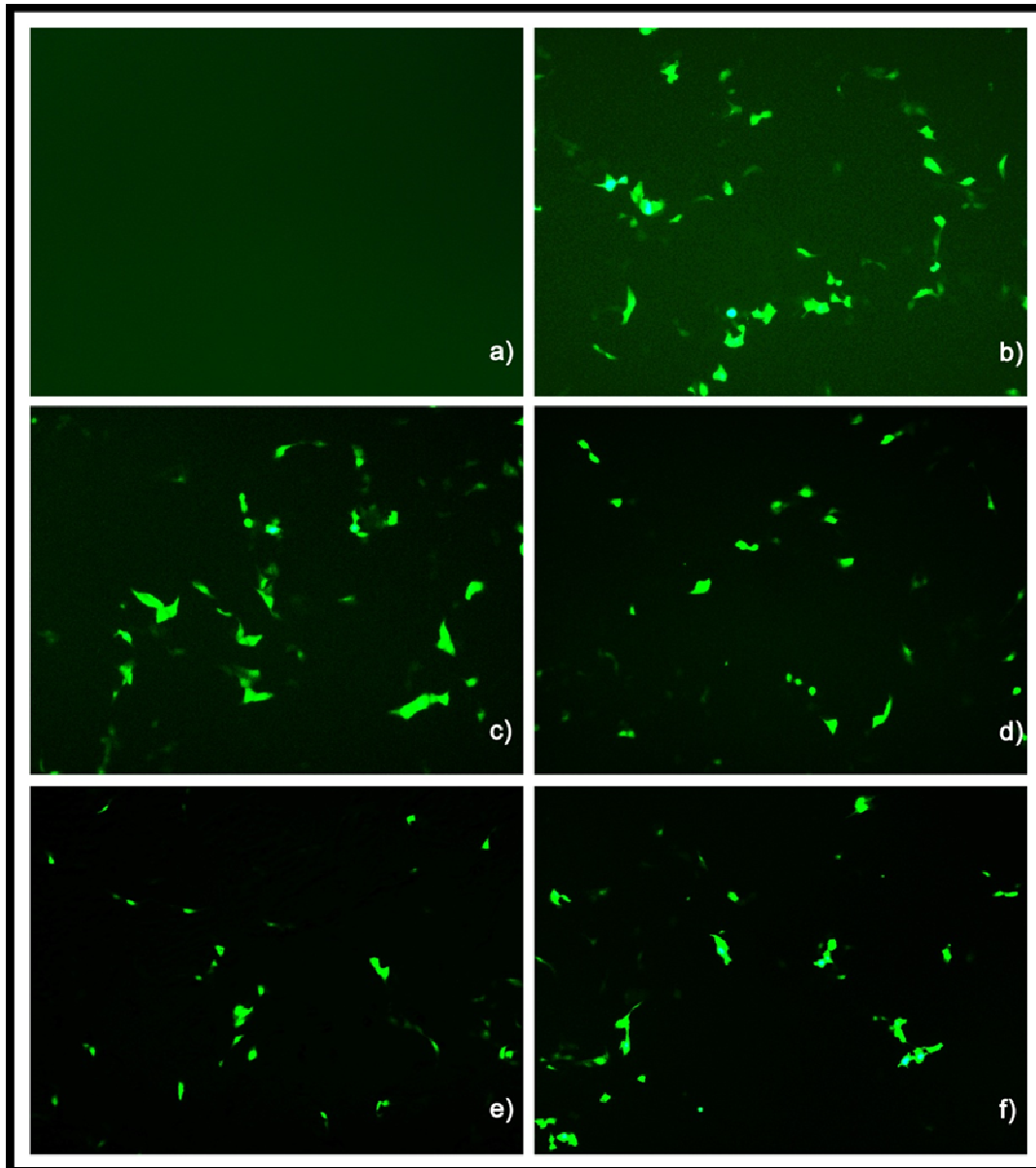
Figure 14: Transfection efficiency of MDCK II cells following transient transfection with the plasmid pGFP-N3 in relation to increasing volumes of transfection Reagent (FuGENE® HD). MDCK II cells were cultured for 24 hours to a confluency of 80-90%. Cells were transfected either without DNA (a) or 2 µg DNA (b-f) in the presence of (b) 8, (c) 9, (d) 10, (e) 11 or (f) 12 µl of FuGENE® HD. All pictures were taken with a 10 X objective using an inverted microscope and an excitation/emission filter set appropriate to detect GFP-fluorescence. A ratio of FuGENE® HD : DNA of 9 : 2 (c) was selected for further transfections.

Establishment of PCR protocols for detection of human MT 1 cDNA

In order to detect human MT 1 (MT1) mRNA expression, either endogenous or following transfection of cells with cDNA encoding for MT1, two different PCR protocols were established in the lab using MT1 specific sense and antisense primers as described by Lanoix ([21]) and Reppert ([57]), respectively. PCR conditions were established using the

plasmids 3xHA-hMT1, flag-hMT1 and hMT1-GFP, all cloned into the mammalian expression vector pcDNA3 as positive controls (4.1.5). The plasmids were obtained and purified as described in (4.1.5) and DNA concentrations and purities were determined as described in (4.3.3). As shown in table 13, all plasmids showed satisfying purity for subsequent transfections ($A_{260/280}$ >1.8).

Table 13: The concentrations of the plasmids in µg/µl (calculated from absorption at 260 nm) and their purity (ratios of absorption at 260nm/280nm and 260nm/230nm) determined with the nanodrop spectrophotometer

Plasmid	Concentration [µg/µl]	Ratio $A_{260/280}$	Ratio $A_{260/230}$
pGFP – N3	0.9	1.87	2.30
hMT 1 HA	0.78	1.88	2.24
hMT 1 flag	1.00	1.91	2.20
hMT 1 GFP	1.20	1.87	2.30

For the amplification of hMT 1 cDNA, each plasmid was diluted 1 : 1000. 1 µl i.e. ~ 1 ng of each plasmid was subjected to a PCR reaction according to 4.5.3 using a set of primers that should produce a PCR product of 286 bp ([57]). In addition, 1 µl of each plasmid dilution was subjected to a PCR reaction according to 4.5.3 using a set of primers that should produce a PCR product of 442 bp ([21]). The resulting PCR products were separated on a 1.5 % native agarose gel according to 4.6 and the results are shown in Figure 15. Negative control PCR reactions, where the cDNA was replaced by water, were performed and did not result in any bands (data not shown) Both PCR conditions resulted in PCR products of expected lengths for all three plasmids, thereby also confirming the identity of the MT1 cDNA insert in the plasmids.
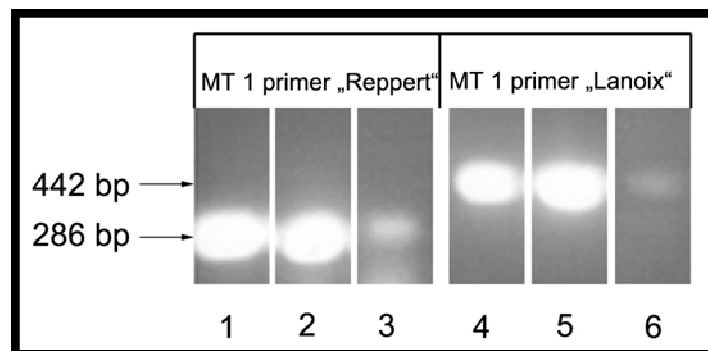
Figure 15: Establishment of the PCR protocols for amplification of human MT1 cDNA. Detection of 286 bp (1-3) and 442 bp (4-6) PCR products following amplification of MT1 cDNA in 3xHA-hMT1 (1, 4), hMT1-GFP (2, 5) and flag-hMT1(3, 6) according to Reppert et al. (1-3) ([57]) or Lanoix et al (4-6) ([21]).

Verification of endogenous expression of hMT1 mRNA in BeWo and MDCK II cells and human placental chorionic tissue by RT-PCR

Endogenous expression of MT1 mRNA in BeWo cells and placental tissue has been demonstrated recently by Lanoix and coworkers ([22]; [21]). However, expression of MT1 mRNA in MDCK II cells has not been investigated. In order to confirm absence or presence of MT1 transcripts in MDCK II cells, RT-PCR was performed. BeWo cells and human placental chorionic tissue as well as HOS and MG63 cells ([98]) were included in this experiment as positive controls.

Total RNA was extracted from all cell lines (BeWo, MDCK II, HOS and MG63) and from placental chorionic tissue according to 4.3. In case of BeWo and MDCK II cells, RNA was isolated from two independent samples. The RNA concentration was determined by measuring the absorption at A_{260} (4.3.3). All RNA samples showed satisfying purity as indicated by determination of the A_{260}/A_{280} and A_{260}/A_{230} ratio (4.3.3). Values of all samples are summarized in table 14.

Table 14: Summary of the concentrations of mRNA in µg/µl (calculated from A_{260}) and mRNA purity (ratios of absorption at $A_{260/230}$ and $A_{260/280}$) isolated from BeWo, MDCK, HOS and MG63 cell lines as well as placental chorionic tissue

#	Cell line	Concentration in µg/µl	Ratio 260/280 nm	Ratio 260/230 nm
1	BeWo (preparation 1)	1.01	1.9	2.08
2	MDCK II (preparation 1)	0.43	1.83	2.14
3	h Placenta	0.46	1.67	2.15
4	HOS	1.10	1.91	2.23
5	MG 63	0.40	1.78	1.77
6	BeWo (preparation 2)	0.15	1.80	1.66
7	MDCK II (preparation 2)	0.56	1.92	1.61

Integrity of the RNA samples was verified by native agarose gel electrophoresis and inspection of 28S and 18S rRNA band integrity (4.6). Respective data are shown in Figure 16a.

2 µg of intact RNA from each sample was then transcribed into the complementary DNA (cDNA) as detailed in 4.4 using random hexamer primers and MultiScribe™ MuLV reverse transcriptase. To control this process, 50 ng cDNA from all reverse transcribed samples were subjected to a subsequent PCR reaction, that aimed at cDNA amplification of the housekeeping gene ß-actin according to Neuchrist and coworkers ([170]) (4.5.3).

Amplification products were analysed by native agarose gel electrophoresis (4.6) and the result is shown in Figure 16b. In all samples, a PCR product of 838 bp corresponding to the expected size of the β-actin fragment was detected, confirming that reverse transcription occurred.

To detect MT1 cDNA, 50 ng cDNA from all samples were employed in a PCR reaction according to 4.5.3 using the "Reppert" set of primers that should produce a PCR product of 286 bp ([57]). In addition, 50 ng cDNA from all samples were subjected to the PCR reaction to amplify MT1 cDNA according to 4.5.3 using the "Lanoix" set of primers that should produce a PCR product of 442 bp ([21]). The PCR products of both reactions were separated on a 1.5 % native agarose gel according to 4.6 and the results are shown in Figure 16 c and d. In all cell lines (BeWo, MDCK, HOS and MG63) as well as in placental chorionic tissue, PCR products of the expected sizes of 286 bp ([57]) and 442 bp ([21]) could be demonstrated. This confirm expression of MT1 mRNA in BeWo cells and placenta as well as HOS and MG 63 cells ([21]; [98]) and provide evidence for MT1 mRNA expression in MDCK II cells. The only exception was one of the two independently prepared BeWo samples, where amplification of a 286 bp PCR product according to ([57]) failed. We repeated isolation of mRNA, and RT-PCR with a third independent BeWo sample and obtained positive results with "Lanoix" as well as "Reppert" PCR reactions (data not shown). It is, however, of interest to note that our recently performed quantitative real time PCR reaction using an assay from Applied Biosystem for amplification (MTNR1A assay, AB Hs00195567_m1), also failed to detect endogenous expression of MT1 in BeWo cells, while expression in human chorionic tissue and TC71-where MT1 expression has been demonstrated before ([98])-was shown.
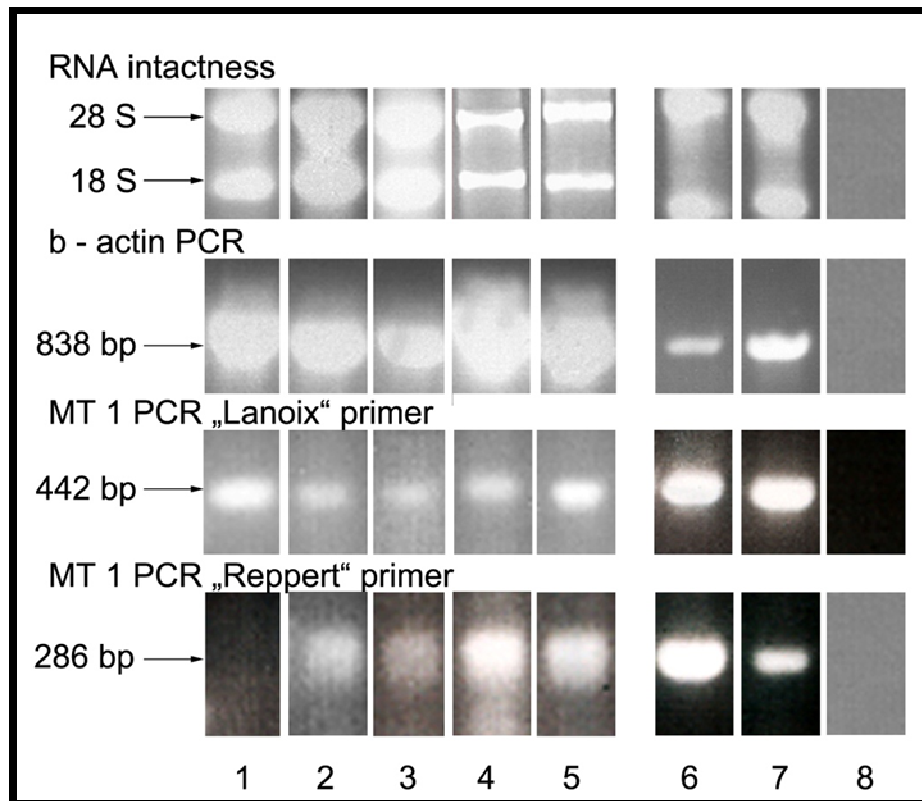
Figure 16: Jugment of integrity of RNA samples extracted from (1) BeWo cells (2) MDCK II cells, (3) human placenta, (4) HOS, (5) MG 63, (6) BeWo 2 and (7) MDCK II 2. Arrows indicate intact 28S and 18S bands. (b) Detection of β-actin transcripts (838 bp) to confirm successful RT reaction. (c) and (d) Detection of MT1 transcripts (442 bp as well as 286 bp) by two different PCR products according to Lanoix and Reppert ([21]; [57])

5.2 Transient and stable transfection of BeWo and MDCK II cells with human MT1 cDNA tagged with either flag or triple HA peptides or the green fluorescent protein (GFP)

5.2.1 Transient transfection of BeWo and MDCK II cells with pCDNA3 containing 3xHA-hMT1, flag-hMT1 or hMT1-GFP

Next, we transfected BeWo as well as MDCK II cells with the plasmids 3xHA-hMT1, flag-hMT1 and hMT1-GFP using the FuGENE® HD transfection reagent and the above established transfection protocol (see 5.1.1). Transfected cells as well as parental cells were plated and after 24 hours, the expression of MT1 mRNA was analysed in both cell lines by RT-PCR according to 5.1.1. The results of this transient transfection are summarized in Figure 17.

Purity (ratios $A_{260/230}$ and $A_{260/280}$) and integrity of the isolated RNA (Figure 17a) were confirmed and 2 µg of all RNA samples were reverse transcribed as detailed above. Formation of intact cDNA was controlled by amplification of a 838 bp PCR product corresponding to the sequence of the ß-actin cDNA in all samples (Figure 17b). Then, 50 ng cDNA from all samples were subjected to the PCR reactions according to Reppert and Lanoix, aiming for amplification of MT1 cDNA fragments of 286 bp ([57]) and 442 bp ([21]), respectively. The PCR products of both reactions were analysed by a native agarose gel electrophoresis (gel 1.5 %) and the results are shown in Figure 17 c ([21]) and d ([57]).

Despite the fact that conventional RT-PCR is not a quantitative method, a significant increase in the amount of the 286 bp as well as the 442 bp band was observed in BeWo as well as MDCK II cells transfected with pcDNA3 vectors containing 3xHA-hMT1 and flag-hMT1 cDNA indicating an increase in the respective MT1 mRNA. Transfection with hMT1-GFP cDNA cloned into pcDNA3 resulted in undetectable to low expression of MT1 mRNA in these cells as judged from the low to undetectable PCR products obtained from either the "Reppert" or the "Lanoix" PCR protocol. Corresponding to the results presented in 5.1.1, endogenous expression of MT1 mRNA was detected in BeWo and MDCK cells at a low level with both primer sets, except for BeWo cells using the "Reppert"-primer pair. In this case, no PCR product was amplified.

The experiment was repeated and transient expression of 3xHA-hMT1, flag-hMT1 and hMT1-GFP mRNA was analysed by RT-PCR with MT1 sequence-specific "Reppert" and "Lanoix" primers and PCR protocols 24 hours and 48 hours after the experiment with similar results (data not shown). While transfection of both BeWo and MDCK II cells with

the vectors encoding 3xHA-hMT1 and flag-hMT1 cDNA resulted in induced expression of MT1 mRNA, cells transfected with hMT1-GFP cDNA exhibited similar low MT1 mRNA levels as the parental cell lines. Since transfection of cells with hMT1-GFP cDNA should in principal result in expression of a fluorescent-tagged MT1 protein, if translation of mRNA into protein had occurred, we investigated the BeWo and MDCK II cells transfected with hMT1-GFP cDNA 24 hours and 48 hours after transfection by direct fluorescence microscopy (4.2). As estimated from the low MT1 mRNA level in these cells, we were not able to detect any significant GFP signal in neither BeWo nor MDCK II cells (data not shown).

In summary, using the lipid-based transfection reagent FuGENE® HD, BeWo cells and MDCK II cells were successfully transfected with the mammalian transfection vector pcDNA3 containing 3xHA-hMT1 and flag-hMT1 as determined by upregulation of MT1 mRNA in comparison to the parental cell lines.
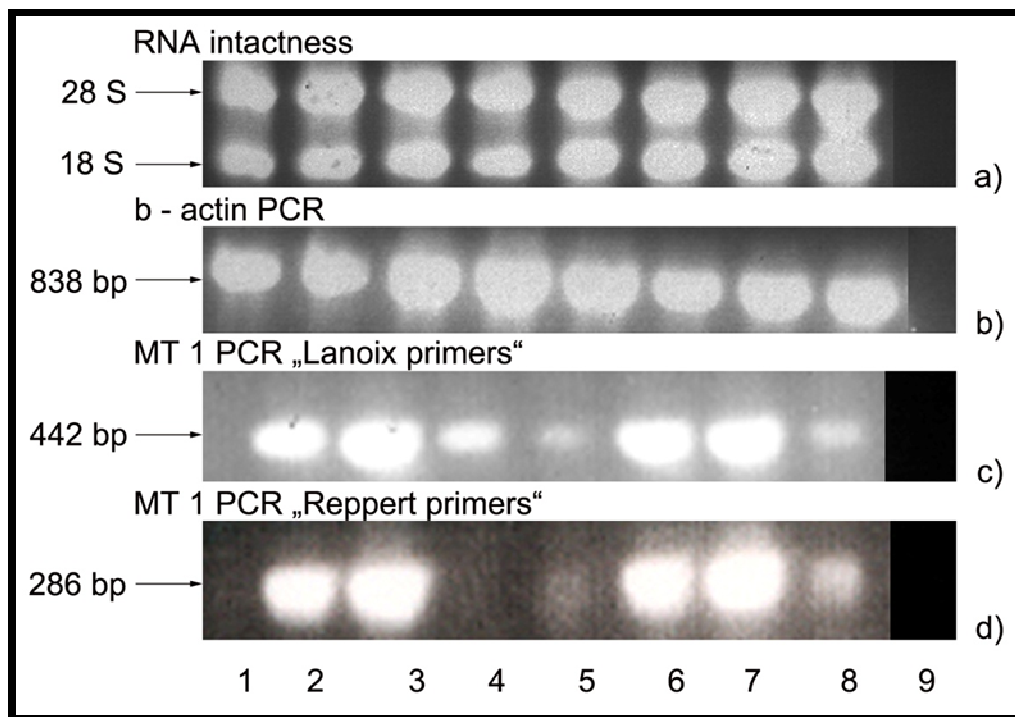
Figure 17: Jugment of integrity of RNA samples extracted from (1) BeWo GFP MT1 (2) BeWo HA MT1, (3) BeWo flag MT1, (4) BeWo parental, (5) MDCK II GFP MT1, (6) MDCK II HA MT1 and (7) MDCK II flag MT1 (9) MDCK II parental. Arrows indicate intact 28S and 18S bands. (b) Detection of β-actin transcripts (838 bp) to confirm successful RT reaction. (c) and (d) Detection of MT1 transcripts (442 bp as well as 286 bp) by two different PCR products according to Lanoix and Reppert ([21]; [57])

5.2.2 Selection of stably transfected clones of BeWo cells following transient transfection with pcDNA3 containing 3xHA-hMT1 and flag-hMT1

In order to generate stably transformed cell lines from the above described transiently transfected BeWo and MDCK II cells, we took advantage of the neomycin resistance gene encoded by the pcDNA3 vector. Only cells that by chance had integrated the transfected plasmid in their genome, should be able to proliferate and survive in the presence of the antibiotic Geneticin (G418) that inhibits protein synthesis of mammalian cell lines. Consequently, 72 hours after transient transfection of BeWo cells with pcDNA3 containing 3xHA-hMT1 and flag-hMT1 cDNA, the medium of the cells was supplemented with 0.25 mg/ml G418, an optimal concentration for selection of transfected BeWo cells as determined earlier ([108]). After approximately 10-14 days cell islands appeared in BeWo cultures that had been transfected with 3xHA-hMT1 and flag-hMT1 cDNA, while in culture dishes with the parental BeWo cells all cells were dead at this time point. These cell islands were then cloned and expanded according to 4.1.6 until enough cells were obtained to generate cell lysates (4.7.4) and freeze cell stocks (4.1.6) for future investigations. Prior freezing, clones were analyzed by microscopy for their appearance. It was of interest to note that some of the BeWo cells transfected with either 3xHA-hMT1 (e.g. clones 5 and 6) or flag-hMT1 (e.g. clones 8 and 10) exhibited a more elongated morphology than the parental BeWo cells (see Figures 18 and 19). To summarize, following successful transient transfection of BeWo cells with either 3xHA-hMT1 and flag-hMT1 cDNA, various G418-resistant cell clones were generated and preserved.
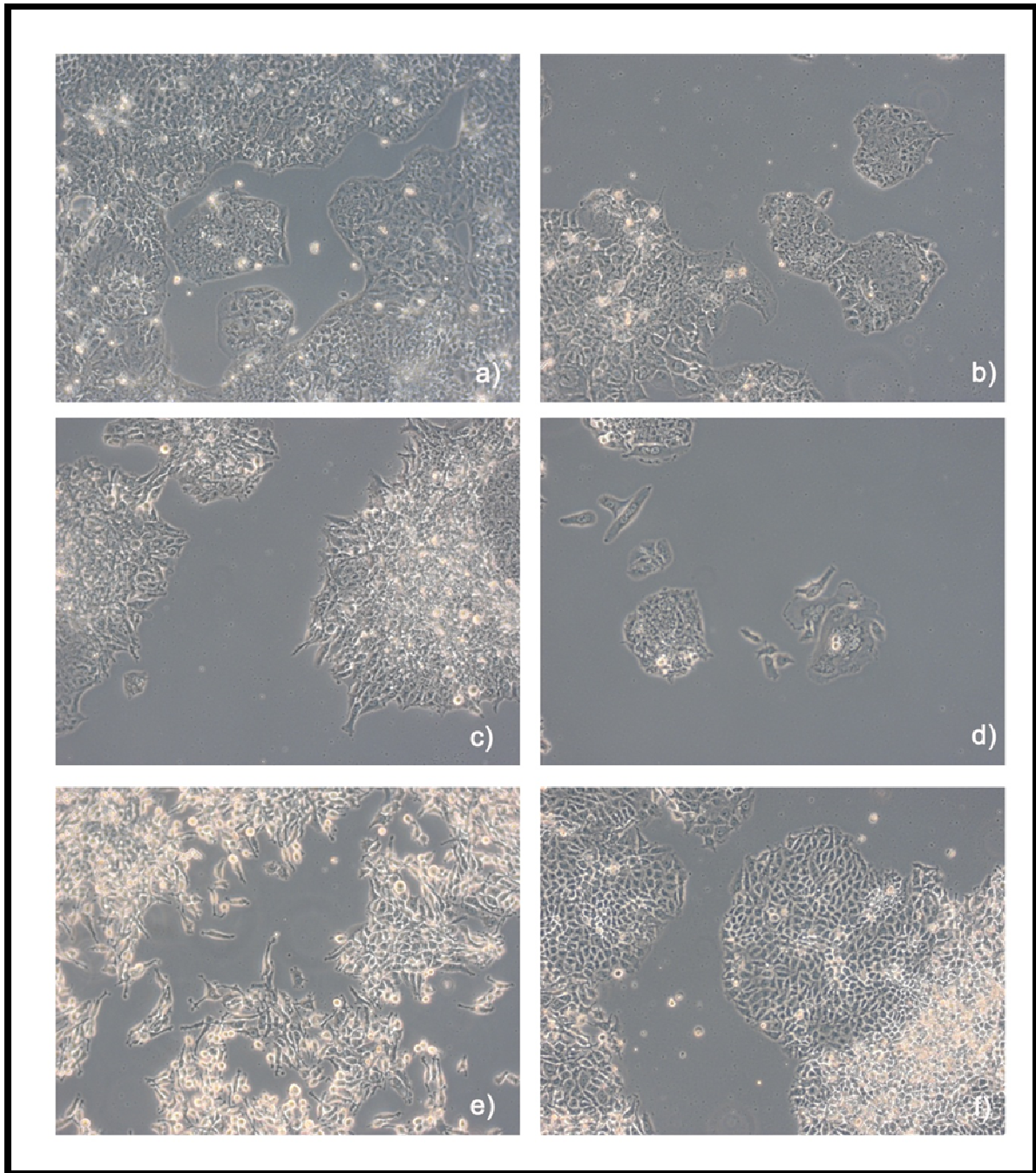
Figure 18: Morphology of parental BeWo and BeWo cells transfected with flag MT1 (a) parental BeWo, (b) clone 3, (c) clone 8, (d) clone 9, (e) clone 10, (f) clone 12. Phase contrast microscopy was done on unfixed cells with a 10x objective. Note elongated cell morphologies in (c), (d) and (e) in comparision to (b).
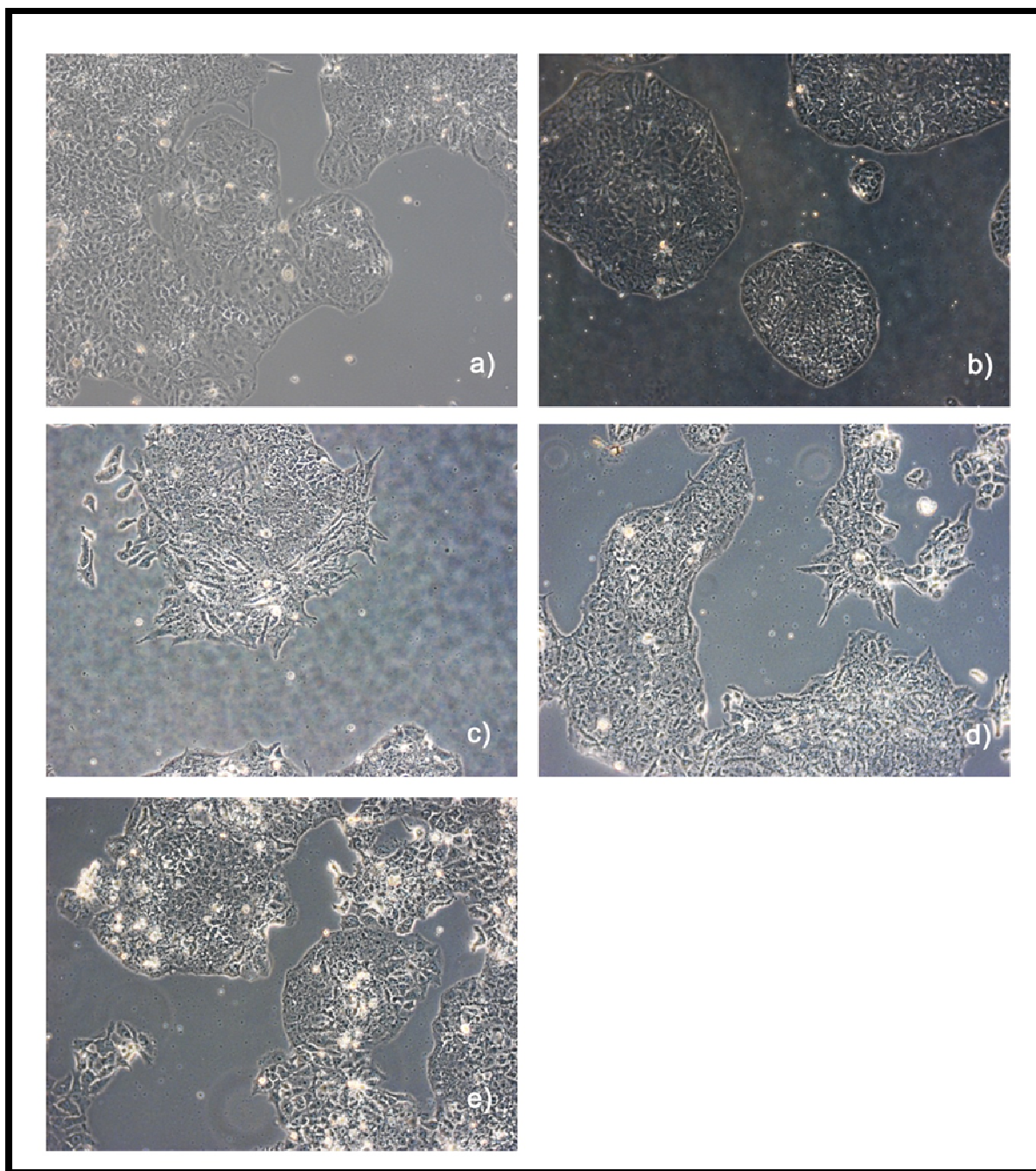
Figure 19: Morphology of parental and BeWo parental cells transfected with HA MT1 (a) BeWo parental (b) clone 1, (c) clone 5, (d) clone 6, (e) clone 10. Pase contrast microscopy was done on unfixed cells with a 10x objective. Note elongated cell morphologies in (c) and (d).

5.3 Characterization of various anti-MT1, anti-flag-tag or anti-HA-tag antibodies by western blotting

In the last set of experiments, we performed western blotting experiments on total cell lysates (4.7.4) prepared from parental BeWo cells and MG63 cells with endogenous expression of MT1 mRNA as well as several G418-resistant cell clones obtained following transfection of parental BeWo cells with either 3xHA-hMT1 or flag-hMT1 cDNA, which resulted in induction of MT1 mRNA expression (5.2). These experiments were conducted to analyze the expression of MT1 protein in these cells to demonstrate the expected increase upon transfection of MT1 cDNA, but also to characterize several anti-MT1 antibodies or anti-tags (flag, HA) antibodies. In all experiments, same amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes as detailed in 4.7.4. Following blocking, the PVDF membranes were incubated with different sets of antibodies combining MT1 or tag-specific primary antibodies with HRP-labeled secondary antibodies as indicated in 4.7.4. Detection of HRP-labeled proteins was then performed as detailed in 4.7.4.

5.3.1 Western blot analysis of BeWo, MG63 and BeWo+flag-MT1 lysates with two anti-MT1 antibodies (Santa Cruz sc-13179 and Abbiotech 250761) and an anti-flag antibody (Sigma F7425)

Samples containing 40 µg protein were loaded onto 10 % gels as and subsequent western blotting as indicated in table 15. Four identical blots were generated and incubated with the antibody combinations indicated in table 16. In figure 20, the blot was incubated with the anti-MT1 antibody sc-13179 from Santa Cruz. As observed in our previous studies ([166]) and by others ([21]; [22]), this antibody detects 1 protein of ~ 40-50 kDa. The protein was detected in parental BeWo cell lysates (lane 1 and 7), MG63 cell lysates (lane 8) and lysates from BeWo cells transfected with flag-MT1 cDNA (BeWo+flag-MT1; lane 2-6). Expression of this protein in the cells was variable, but not upregulated following transfection with flag-MT1 cDNA. The variabilities in expression of the detected protein were not due to loading differences as was confirmed by stripping and reincubation of this blot with an anti-tubulin antibody (data not shown; 4.7.4). Preincubation of the primary anti-MT1 antibody sc-13179 with a corresponding blocking peptide (4.7.4) inhibited binding of the antibody to membrane 2, demonstrating specific binding of the antibody to the membrane (Figure 21). In contrast, a completely different result was obtained, when blot 3 was incubated with the anti-MT1 antibody from Abbiotech (250761). Instead of one band, a variety of bands was detected (Figure 22),

ranging from low molecular weight bands to bands of more than 70 kDa, however, none of these bands was significantly increased following transfection of BeWo cells with flag-MT1 cDNA (lane 2-6 versus lane 1 and 7). Reduction of antibody concentration to improve specificity, resulted in a general loss of signal (data not shown). Finally, blot 4 was incubated with an anti-flag antibody to detect overexpressed flag-tagged MT1 proteins (Figure 23). Several protein bands reacted with the antibody, but none of these bands was specifically found in lysates of BeWo+flag-MT1 cells (lane 2-6). Again, a reduction of the antibody concentration to improve specificity resulted in a general loss of signal (data not shown).

We concluded from this set of experiment that none of the BeWo cell clones stably transfected with flag-MT1 cDNA overexpressed a flag-tagged (MT1) protein dispite the observed increase in MT 1 mRNA. Two different anti-MT1 antibodies reacted with proteins of different molecular weight by western blotting. While the Santa Cruz antibody (SC-13179) recognized a 40 kDa protein that could either represent a non-glycosylated MT1 or another, yet unidentified protein, the anti-MT1 antibody from Abbiotech recognized multiple proteins, which is not of advantage when this antibody should be applied in microscopy.

Table 15: Alignment of parental and transfected (flag MT1) BeWo cells

Sample #	1	2	3	4	5	6	7	8
Marker	BeWo parental 1	BeWo flag MT1 clone 3	BeWo flag MT1 clone 8	BeWo flag MT1 clone 9	BeWo flag MT1 clone 10	BeWo flag MT 1 clone 12	BeWo parental 2	MG 63

Table 16: Combination of antibodies used in Figure 20 and 23

AB pair	Antigen	Primary AB	Company/number	Secondary AB	Company/number
1a) Fig 20	hMT 1	Goat anti hMT1 1 : 200	Santa Cruz SC 13179	Donkey anti goat IgG HRP 1 : 5000	Santa Cruz SC 2020
1b) Fig 21		+ blocking peptide	SC 13179 P	Donkey anti goat IgG HRP	Santa Cruz SC 2020
2 Fig 22	hMT1	Rabbit anti hMT1 1 : 100	Abbiotech 250761	Goat anti rabbit IgG HRP 1 : 16000	Sigma A0545
3 Fig 23	Flag	Rabbit anti flag 1 : 100	Sigma F7425	Goat anti rabbit IgG HRP 1 : 16000	Sigma A0545
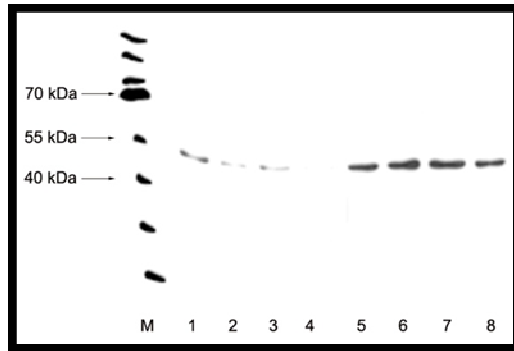
Figure 20: Western blot analysis of MT1 in samples 1-8 defined in table 15. Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with antibody pair 1a), containing anti MT1 antibody from Santa Cruz. The blot was exposed to the film for 5 minutes.
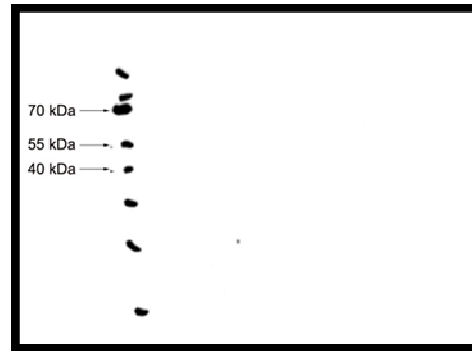
Figure 21: Western blot analysis of MT1 in samples 1-8 defined in table 15. Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with antibody pair 1b), containing anti MT1 antibody from Santa Cruz and blocking peptide. The blot was exposed to the film for 5 minutes.
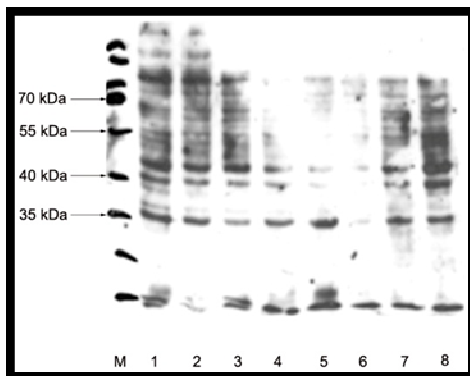
Figure 22: Western blot analysis of MT1 in samples 1-8 defined in table 15. Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with antibody pair 2), containing anti MT1 antibody from Abbiotech 250761. The blot was exposed to the film for 1 hour.
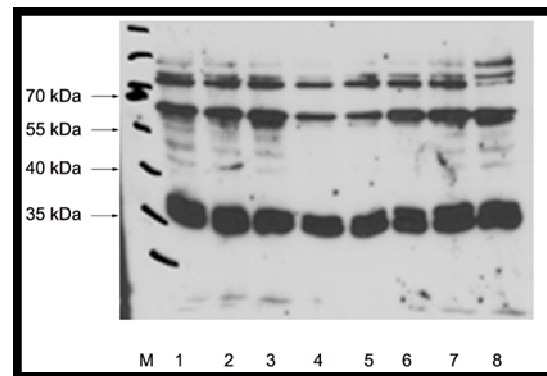
Figure 23: Western blot analysis of MT1 in samples 1-8 defined in table 15. Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with antibody pair 3), containing anti MT1 antibody from Sigma F7425. The blot was exposed to the film for 1 hour.

5.3.2 Western blot analysis of BeWo and BeWo+3xHA-MT1 lysates with the anti-MT1 antibody from Santa Cruz (sc-13179) and an anti-HA antibody from Roche (867-423-001)

Again, samples containing 40 µg protein were loaded onto 10 % gels as indicated in table 17 and therafter western blots were prepared. Two identical blots were generated and incubated with the antibody combinations indicated in table 18. As in figure 24, the first blot was incubated with the anti-MT1 antibody sc-13179 from Santa Cruz. As seen before, this antibody detected one protein of ~40-50 kDa. This protein was found in parental BeWo cell lysates (lane 1 and 9), and in lysates from BeWo cells transfected with 3xHA-MT1 cDNA (BeWo+3xHA-MT1, lanes 2-8). Like before (5.3.1), expression levels of this protein in the cells was variable, but not upregulated following transfection with 3xHA-MT1 cDNA. We controled for and excluded loading differences in the protein amount by stripping and reincubation of this blot with an anti-tubulin antibody (data not shown). The second blot was incubated with an anti-HA antibody to detect overexpressed HA-tagged MT1 proteins (figure 25). The antibody did not react with any protein on the blot, even if the antibody concentration was increased and the exposure time prolonged, suggesting that the lysates contained no protein with a HA-tag.

To summarize this set of experiments: none of the BeWo cell clones stably transfected with 3xHA-MT1 cDNA was found to overexpress a HA-tagged (MT1) protein. Again, the Santa Cruz antibody recognized a 40 kDa protein that could either represent a non-glycosylated MT1 or another, yet unidentified protein.

Table 17: Alignment of parental and transfected (3x HA MT1) BeWo cells

|	1	2	3	4	5	6	7	8	9
Marker	BeWo parental 1	BeWo HA MT1 clone12	BeWo HA MT1 clone 13	BeWo HA MT1 clone 14	BeWo HA MT1 clone 15	BeWo HA MT1 clone 16	BeWo HA MT1 clone 17	BeWo HA MT1 clone 18	BeWo parental 2

Table 18: Combination of antibodies used in Figure 24 and 25

| AB pair	Antigen	Primary AB	Company/number	Secondary AB	Company/number
1 Fig 24	hMT1	Goat anti hMT1 1:200	Santa Cruz SC 13179	Donkey anti goat IgG HRP 1: 5000	Santa Cruz SC 2020
2 Fig 25	HA	Rat anti HA 1000	Roche 867-423-001	Goat anti rat IgG HRP 1:5000	Jackson Immuno Research; 112-035-003
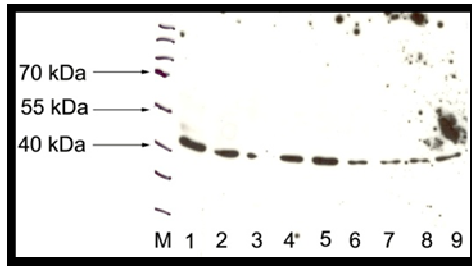
Figure 24: Western blot analysis of MT1 in samples 1-9 defined in table 15. Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with antibody pair 1), containing anti MT1 antibody from Santa Cruz (SC 13179). The blot was exposed to the film for 1 hour.
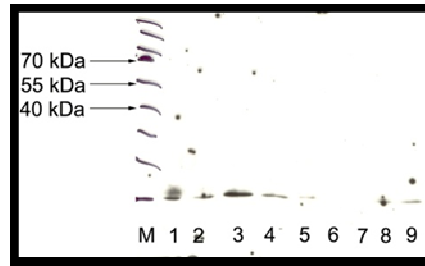
Figure 25: Western blot analysis of MT1 in samples 1-9 defined in table 15. Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with antibody pair 2), containing anti MT1 antibody from Roche 867-423-001. The blot was exposed to the film for 1 hour

5.3.3 Western blot analysis of BeWo, BeWo+flag-MT1 and BeWo+3xHA-MT1 lysates with the anti-MT1 antibody from Santa Cruz (sc-13179) and an anti-MT1 antibody prepared by ([93])

In this last set of experiments, we wanted to compare the antigenicity of two anti-MT1 antibodies, namely sc-13179 derived from Santa Cruz (directed against the N-terminal region of MT1), and an anti-peptide antibody generated by ([93]) against the c-terminal amino acids 341-350 of human MT1. As before, samples containing 40 µg protein were loaded onto 10 % gels as indicated in table 19 and. Thereafter, western blots were performed. One membrane with two identical halves was generated and, following blocking, cut into two pieces. These two pieces were incubated with the respective antibody combinations indicated in table 20. The result is seen in figure 26. The left side of the blot was incubated with anti-MT1 antibody sc-13179 from Santa Cruz. As usual, this antibody detected one protein of ~40-50 kDa. This protein was found in parental BeWo cell lysates (lane 1), and in lysates from BeWo cells transfected with either 3xHA-MT1 cDNA (BeWo+3xHA-MT1, lanes 5 and 6) or flag-MT1 cDNA (BeWo+flag-MT1, lane 7). Levels of expression of this protein in the cells varied, but loading differences were excluded by stripping and reincubation of this blot with an anti-tubulin antibody (data not shown). The second half of the blot with identical samples was incubated with the anti-MT1 antibody derived from Angeloni and coworkers ([93]). This antibody gave one major

band with an approximate molecular weight of 55-60 kDa and two less intense bands of higher molecular weight (more than 80 kDa). Of importance, the molecular weight of the smallest protein detected by Angelonis´s antibody was clearly larger than the protein detected by anti MT1 sc-13179 from Santa Cruz.

In conclusion, a polyclonal antibody prepared against a peptide corresponding to the c-terminal amino acids 341-350 of human MT1 recognized one major protein in all cell lysates investigated that had a molecular weight resembling that of the suggested molecular weight of the glycosylated MT1 receptor. This is in contrast to the anti-MT1 antibody from Santa Cruz that always detects a protein at ~40-50 kDa that could represent a non-glycosylated form of the protein. This antibody is directed against the N-terminal domain of MT1. Clearly, all three anti-MT1 antibody tested in this study detect different proteins in cell lysates with demonstrated expression of MT1 mRNA. Since the human MT1 protein is most likely a glycosylated protein of ~ 60kDa, it must be concluded that the antibody provided by Angeloni et al. ([93]) currently, represents the best candidate for further investigations of MT1 protein.

Table 19: Alignment of parental and transfected (3x HA MT1; flag MT1) BeWo cells

Blot 3	Old antibodies				Blot 4	New antibodies (Fraschini)			
	1	5	6	7		1	5	6	7
Marker	BeWo Parental 1	BeWo HA MT1 clone 11	BeWo HA MT1 clone 15	BeWo Flag MT1 clone 12	Marker	BeWo parental 1	BeWo HA MT1 clone 11	BeWo HA MT1 clone 15	BeWo Flag MT1 clone 12

Table 20: The used antibodies, companies and numbers

AB pair	Antigen	Primary AB	Company/number	Secondary AB	Company/number
1	hMT 1	Goat anti hMT 1	Santa Cruz SC 13179	Donkey anti goat IgG HRP	Santa Cruz SC 2020
2		Rabbit α human MT 1	Angeloni et al. [93]	Goat anti rat IgG HRP	Sigma A0545
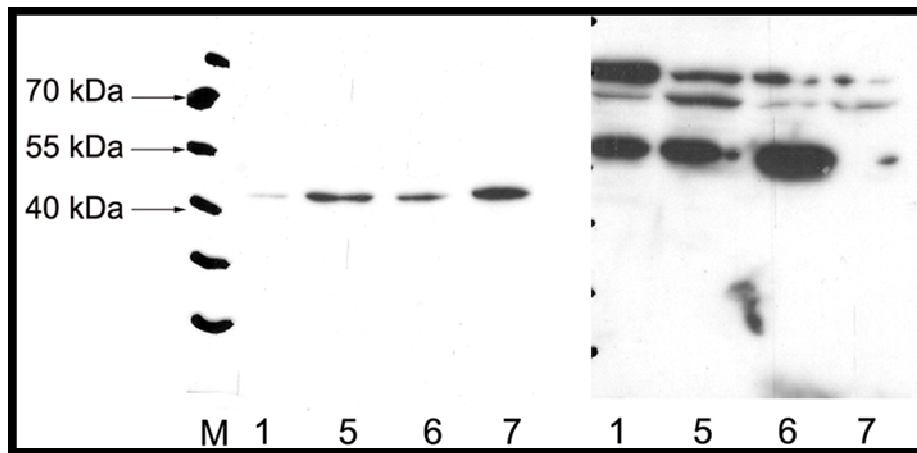
Figure 26: Western blot analysis of MT1 in samples 1, 5, 6 and 7 defined in table 15. Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to a PVDF membrane. The left side of the membrane was incubated with antibody pair 1), containing anti MT1 antibody from Santa Cruz (SC 13179). The right side was incubated with antibody pair 2 containing anti MT1 from Angeloni ([93]). The blot on the left side was exposed to the film for 30 min,whereas the blot on the right sidewas exposed to the film for 20 sec

6 Discussion

Increased levels of melatonin during human pregnancy ([115]; [116]; [19]), a likely production of melatonin in the human placenta ([22]) and the expression of receptors for melatonin (MT1, MT1, ROR; ([21] and [22]) have been demonstrated in recent years. In conjunction with other studies, that had suggested an important role of melatonin in the regulation of trophoblast development and their endocrine function ([135]; [20]; [22]), it seems to be of importance to obtain a complete picture of receptor-mediated melatonin functions in human placental trophoblast cells. The suggested treatment of the pregnancy-related and oxidative stress-associated disease preeclampsia with the radical scavenger melatonin (in pharmaceutical dosis; ([36]) furthermore requests characterization of receptor-mediated melatonin functions.

One major aim of this study was therefore to establish an *in vitro* system that allows for better characterization of MT1 functions in trophoblast-derived cells. For that purpose, we chose the choriocarcinoma cell line BeWo that exhibits many trophoblast-like characteristics, can be cultured as a tight polarized epithelial monolayer ([109]; [107]) and, most importantly, shows endogenous expression of MT1 mRNA. This has been demonstrated by ([21]), and has been confirmed in this study. A major drawback when investigating MT1 functions, however, is the lack of well-characterized antibodies that are needed for a better understanding of MT1 protein expression and localization. Characterization of such antibodies can be best performed on cell lines with increased expression of the antigen. An increase in protein expression also contributes to the characterization of protein function as has been demonstrated for the neonatal Fc receptor FcRn in BeWo cells ([108]), for the polymeric IgA receptor in MDCK II cells ([162]) or for human MT1 in COS-7 cells ([164]; [60]; [165]).

In order to increase the expression levels of MT1 in BeWo cells, but also MDCK II cells (a well-known model system for polarized epithelial cells), we established a lipid-based transfection protocol to transform these cells with three different human MT1 containing plasmids. The MT1 cDNA in these plasmids was either N-terminally linked with a flag-tag (flag-MT1; [164]) or triple HA-tag (3xHA-MT1; [165]) or c-terminally with GFP (MT1-GFP [60]). All three plasmids have been successfully used to study MT1 protein functions in cell culture systems ([60]; [164]; [165]). We indeed succeeded in transient transfection of flag-MT1 and 3xHA-MT1 and a subsequent MT1 mRNA raise in BeWo as well as MDCK cells, however, failed to elevate MT1 mRNA levels after transfection with the MT1-GFP construct. BeWo cells with increased mRNA levels were then selected in the presence of geneticin to obtain stable transfected cell lines. Unfortunately, these clones - although

resistant to the antibiotic geneticin, which indicates genomic integration of the transfected plasmid - did not exhibit any significant increase in MT1 protein expression as tested with the tag-specific antibodies anti-HA and anti-flag by western blotting. In our experience, such stable transfected cell clones are of great advantage for characterization of proteins such as the FcRn ([177]; [108]). However, most of the studies that served to characterize MT1 protein have been performed following transient transfection of the MT1 constructs into non-polarized COS-7 cells ([165]; [60]; [164]) or CHO cells (Brydon et al., 1999). It therefore remains to be tested whether high levels of flag-tagged or HA-tagged protein would be observed in BeWo and MDCK II cells immediately after transfection and later get downregulated or whether high expression of MT1 protein in the epithelial cell lines BeWo and MDCK II is always (also immediately after transient transfection) limited by endogenous factors.

Another intention of this study was a comparison of various anti-human MT1 antibodies in western blotting experiments, in order to better characterize the antigenicity of available antibodies. As shown in the introduction, in most of the studies that have investigated human MT1 protein expression sofar, a small set of antibodies was used. The polyclonal anti-MT1 antibody from CIDtech Research used in many studies ([91]; [90]; [86]; [89]; [81]) reproducible detected a 37 kDa protein. However, this antibody is no longer available. A polyclonal antibody against the TIL3 peptide (a peptide in the third intracellular loop of the human MT1) also identified a 37 kDa protein and appeared to interact non-specifically with a 66 kDa protein ([77]; [92]). In line with these results, a polyclonal antibody against the N-terminal domain of hMT1 from Santa Cruz detected a 40 kDa protein ([21]; [22]; [166] and this study). In contrast, an antibody generated by Brydon et al ([61]) against the c-terminal domain of the human MT1, detected a glycosylated protein of 60 kDa upon transient transfection of COS cells with MT1 cDNA. The predicted molecular weight of human MT1 according to its amino acid sequence is 40 kDa, which would be in line with the results of the first three antibodies. However, MT1 bears two glycosylation sites in the extracellular domain and almost all G-protein coupled receptors are hydrophic and glycosylated. Considering such post-translational modifications, a higher molecular weight of the human MT1 in agreement with the results of ([61]) seems more likely. In our study we investigated three anti-MT1 antibodies from different sources. We tested the antibodies by western blotting on BeWo cell lysates that have been demonstrated to express MT1 mRNA. An antibody from Abbiotech (250761), directed against the central region of MT1 reacted with many bands in the cell lysates. The Santa Cruz antibody sc-13179 reacted with one protein of 40 kDa, in accordance with the literature ([21]; [22]). We then also tested an antibody produced by Angeloni and coworkers ([93]) that reacts with the c-terminal region of MT1. This antibody mainly reacted with a 55-60 kDa protein and

showed additional reaction with two proteins of higher molecular weight. To our knowledge, this antibody has not been tested in western blotting experiments before. From the combined results, it seems that most of the antibodies directed against the N-terminal region or against sequences in the intracellular loops of MT1 detect proteins with a molecular weight of around 40 kDa. It might be speculated that these antibodies can only react with non-glycosylated forms of MT1, maybe due to sterical hindrance. However, none of these antibodies has been tested on cell lysates overexpressing human MT1, and therefore their specificity for MT1 remains to be tested. In contrast, antibodies directed towards the c-terminal region of MT1 ([61]; [93]) react with a 55-60 kDa protein. This could represent the glycosylated receptor, and at least the antibody generated by Brydon et al ([61]), has confirmed its specificity on MT1 overexpressing cell lysates. It must be concluded that among the three anti-MT1 antibodies tested in this study, the antibody generated by Angeloni et al. ([93]) is probably the most specific, as it detects a protein of around 55-60 kDa, which would be in agreement with the result of Brydon et al. ([61]).

7 Conclusions

This study aimed to establish an *in vitro* model for trophoblast cells with endogenous and induced expression of human melatonin receptor 1 (MT1) to serve as a tool for characterization of (1) MT1 function in human placental trophoblast cells and (2) anti-MT1 antibodies. The trophoblast-derived choriocarcinoma cell line BeWo was selected, endogenous expression of MT1 at the mRNA level confirmed, and a lipid-based transfection protocol with MT1 cDNA containing plasmids was established, that resulted in (transient) upregulation of MT1 mRNA in BeWo cells. In addition, three different anti-MT1 antibodies were tested by western blotting on BeWo cell lysates for their ability to specifically detect MT1 protein. Two antibodies were selected that recognized either only a 40 kDa or mainly a 60 kDa protein. These proteins might represent the non-glycosylated (40 kDa) or the glycosylated (60 kDa) form of human MT1, respectively, and especially the second antibody can therefore be of major interest for future investigations. This established *in vitro* model will give the opportunity to investigate preeclampsia in more detail and the relevance of the hormone melatonin to the occurrence and progression of this disease.

8 List of abbreviation

5 - ML	5 - methoxytryptophol
5 - MT	5 - methoxytryptamine
5 - MTAA	5 - methoxyindoleacetic acid
AA - NAT	arylalkylamine N - acetyltransferase
AD	Alzheimer´s disease
AFMK	N^1 - acetyl - N^2 - formyl - 5 - methoxykynuramine
AMK	N^1 - acetyl - 5 - methoxykynuramine
AMP	adenosine - 5' - monophosphoric acid; 5' - adenylic acid
AMPS	ammoniumpersulfate
AP - 1	activator protein - 1
ATP	adenosine triphosphate
C3 - OHM	cyclic 3 hydroxymelatonin
Ca^{2+}	calcium
CaM kinase II	calcium/calmodulin - dependent protein kinase
cAMP	cyclic adenosine - 5' - monophosphoric acid
CAT	catalase
cDNA	complementary desoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CL	chemoluminescence
Conc.	concentration
COX - 2	cyclo - oxygenase 2
CREB	Ca^{2+}/cAMP response element binding proteins
CTB	cytotrophoblast
CuZnSOD	copper/zinc superoxide dismutase
CYP 450	Cytochrome P450
Da	dalton
DEPC	diethylpyrocarbonate
DMEM	dulbecco´s modified eagle´s medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynucleosidetriphosphate
ECM	extracellular matrix component
EDTA	ethylenediamine tetraacetic acid
ERK	extracellular - signal regulated kinase
EtBr	ethidium bromide
ETC	electron transport chain
EtOH	ethanol
FCS	fetal calf serum
FSH	follicle - stimulating hormone
g	gravity, gram
G418	geneticin
G6PD	glucose - 6 - phosphate dehydrogenase

GFP	green fluorescent protein
GMP	guanosine monophosphate
GnRH	gonadotropin - releasing hormone
GPx/GSH - Px	glutathione peroxidase
GRd	glutathione reductase
GSH	glutathione
GTC	guanidinium thiocyanate
H_2O_2	Hydrogen peroxide
HA	hemagglutinin
hBMSC	human bone marrow stromal cells
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
hFcRn	human neonatal Fc receptor
HIOMT	Hydroxyindole - O - methyltransferase
HME	HEPES - $MgCl_2$ - EDTA
hMT 1	human melatonin receptor MT 1
hOB	human osteoblast
IF	immunofluorescence
IgG	immuneglobuline G
iNOS	inducible nitric oxide synthase
JNK	c - Jun N - terminal kinase
kDa	kilo Dalton
KIR 3	inwardly rectifying potassium channels
LH	luteinizing hormone
LNCaP	human prostate carcinoma cell line
LOO·	peroxyl radical
LPS	lipopolysaccharide
MAO A	monoamine oxigenase A
MAPK	mitogen - activated protein kinase
MEK	MAPK/ERK kinase
Mel 1a, MT1	melatonin receptor 1
Mel 1b, MT2	melatonin receptor 2
MnSOD	manganese superoxide dismutase
MT	melatonin
MTP	mitochondrial transition pore
MUPP 1	Multi-PDZ Domain Protein 1
NAD^+	Nicotinamide adenine dinucleotide
NADH	reduced form of NAD^+
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of $NADP^+$
NAT	N - acetyltransferase
NE	norepinephrin
NF-κB	nuclear factor kappa B
NIH 3T3 cells	mouse embryonic fibroblast cell line
NO	nitrogen monooxide

OH·	hydroxyl radical
ONOO⁻	peroxynitrite anion
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered solution
PCR	polymerase chain reaction
PCS	Pancreatic stellate cells
PE	preeclampsia
PgE_2	prstaglandin E_2
PKC	phospholipase C
PSA	prostate - specific antigen
PSN	Penicillin Streptomycin Neomycin
PTX	pertussis toxin
PVDF membrane	polyvinylidene fluoride
QR 2	quinone reductase 2
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor κ B ligand
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROR	retinoid orphan receptor
RORE	ROR response elements
ROS	reactive oxygen species
RT	reverse transcription
rt	room temperature
RT - PCR	reverse transcription - polymerase chain reaction
RZR	retinoid Z receptor
SCN	superchiasmatic nucleus
SDS	Sodium dodecylsulfate
SDS - PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
STB	syncythiotrophoblast
STB	syncytiotrophoblast
TAE	Tris acetate EDTA
TEMED	tetramethylethylendiamin
Tris	hydroxymethyl aminoethan
v/v	volume per volume
Vol	volume
w/v	weight per volume
x g	amount of accelerations quoted in multiples of g
γ - GCS	γ glutamylcysteine synthetase
IRS4	insulin receptor substrate 4

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