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Expression of melatonin receptor MT-1 in
the BeWo choriocarcinoma cell line

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2 ABSTRACT

2.1 Abstract (English)

Background: Melatonin (MT), the “hormone of the darkness”, is secreted during the night by the pineal gland and is rapidly distributed to many body fluids and tissues to exert a plethora of functions. Major functions of MT in mammals are mediated by two G-protein-coupled membrane melatonin receptors, MT1 and MT2, respectively. Via its receptors, MT functions as a chronobiotic, promotes sleep, shows immunomodulatory and oncostatic effects. Best investigated is the function of MT for the synchronization of the reproductive response to appropriate environmental conditions in photoperiodic animals. MT can cross the human placenta thereby mediating important photoperiodic information to the fetus. MT via receptor-mediated actions also plays an important role in the regulation of human placental cell development and endocrine function, but these effects have not been investigated systematically. **Aim:** To elucidate the function of MT and its receptors in the placenta, three commercially available antibodies against MT1, namely sc-13179, ab-13035 and ab-13036, were tested for their applicability of available anti-MT1 antibody, in western blotting experiments (WBE) and immunofluorescence microscopy (IFM) in BeWo and other cell lines with endogenous expression of MT1. 2/ Stable transfection of BeWo cells with the cDNA encoding for the HA-tagged human MT1 receptor was done to generate an MT1-overexpressing cell line which could be used for further investigation on the effects of the hormone **Results:** All antibodies reacted positively with MT1 mRNA expressing human cell lines and tissues in WBE and IFM, but detected proteins of diverse molecular weights. In good agreement with the literature, sc-13179 detected a protein of approximately 40 kDa in parental BeWo cell and placental tissue extracts by WBE, and revealed a predominant intracellular localization of MT1 in BeWo cells by IFM. Upon transfection of BeWo cells with the cDNA for a HA-tagged human MT1 receptor, several clones were selected that exhibited a strong increase in MT1 mRNA. While the size (40 kDa) and the amount of protein detected by sc-13179 were not significantly altered in these clones in comparison to the parental BeWo cell line, an anti-HA-tag antibody detected the presence of a ~70 kDa protein in the transfected clones, but not the parental cell line. **Discussion:** Although the deglycosylated MT1 core protein has a predicted molecular mass of approximately 40 kDa, because of glycosylation, proteins will migrate with a greater molecular mass. Detection of a 40 kDa protein in BeWo cell lysates therefore suggests that BeWo cells express only a non-glycosylated MT1 receptor. Alternatively, the glycosylated receptor is not detected by sc-13179, explainable by the proximity of the N-glycosylation sites of MT1 and the target sequence of sc-13179. Finally, sc-13179 might not detect MT1 at all, but recognizes a protein sharing sequence homologies with MT1. The results obtained with BeWo cells overexpressing the HA-tagged MT1 indicate that (HA-) MT1 is indeed a glycosylated protein

with a molecular weight of about 70 kDa and support the hypothesis that sc-13179 either not recognizes glycosylated MT1 receptor or identifies an unknown protein with sequence homologies to MT1. In summary, sc-13179 appears not suitable to investigate MT1 protein functions in BeWo choriocarcinoma cells. **Conclusion:** Further studies on the expression of MT1 in the placenta are necessary, but should be done with novel antibodies. These studies are important to evaluate MT1 as a potential target for the treatment of diseases will altered circadian rhythm during pregnancy.

2.2 Abstract (Deutsch)

Hintergrund: Melatonin (MT), das "Hormon der Finsternis", wird während der Nacht von der Zirbeldrüse sezerniert. Nach seiner Verteilung in Körperflüssigkeiten und Geweben übt es eine Vielfalt von Funktionen aus, die durch zwei G-Protein-gekoppelte Membran-Rezeptoren, MT1 and MT2, vermittelt werden. MT kann z.B. chronobiotisch wirken, Schlaf fördern, immunmodulatorische und onkostatistische Effekte ausüben. MT beeinflusst auch die Reproduktion, und bewirkt eine Synchronisation der Reproduktion mit den Umweltbedingungen bei Tieren. Über die Plazenta gelangt MT zum Fötus und vermittelt ihm so wichtige photoperiodische Informationen. Schließlich beeinflusst MT über seine Rezeptoren auch die Entwicklung von plazentaren Trophoblasten sowie deren Hormonproduktion. Diese Funktionen wurden aber bis dato nicht systematisch untersucht

Ziel: Um Untersuchungen über die Expression und Funktion von MT1 in der Plazenta durchführen zu können, sollte zunächst drei kommerziell verfügbaren anti-MT1 Antikörper, sc-13179, ab-13035 und ab-13036, in Westernblot-Experimenten (WBE) sowie Immunfluoreszenz-Mikroskopie (IFM) in BeWo und anderen Zelllinien mit endogener Expression von MT1 charakterisiert werden. Stabile Transfektionen von BeWo Zellen mit einem HA-markierten humanen MT1 Rezeptor sollte eine MT1-überexprimierende Zelllinie generiert werden, die für weitere Untersuchungen an der Plazenta zur Verfügung steht.

Resultate: Alle Antikörper reagierten positiv mit MT1 mRNA exprimierenden humanen Zelllinien und Geweben in WBE und IFM, allerdings zeigten die detektierten Proteine sehr unterschiedliche Molekulargewichte. In guter Übereinstimmung mit der Literatur detektierte sc-13179 ein Protein von ungefähr 40 kDa in parentalen BeWo Zellen und plazentaren Gewebsextrakten in WBE, und es zeigte sich eine vor allem intrazelluläre Lokalisation von MT1 in BeWo Zellen in IFM. Nach Transfektion der BeWo Zellen mit der cDNA für einen HA-markierten humanen MT1 Rezeptor, wurden mehrere Klone mit starker Erhöhung der MT1 mRNA selektiert. Während die Größe (40 kDa) und Menge an Protein, welches von sc-13179 detektiert wurde, sich nicht signifikant gegenüber den parentalen BeWo Zellen veränderte, konnte mithilfe eines anti-HA-Antikörpers der Nachweis eines ~70 kDa Protein in den transfektierten Zellklonen, nicht aber in der parentalen Zelllinie erbracht werden.

Diskussion Das deglykosilierte MT1 Kernprotein hat eine molekulare Masse von circa 40 kDa, wobei aber Glykosylierung zu einem höheren Molekulargewicht führt. Die Detektion eines 40 kDa Protein in BeWo Zelllysaten deutet daher an, dass BeWo Zellen entweder nur die nicht-glykosilierte Form des MT1 Rezeptor expremieren, oder aber, dass sc-13179 den glykosilierten Rezeptor nicht erkennt. Letzteres wäre durch die Nähe von Glykosilierungsstellen und Erkennungssequenz von sc-13179 erklärbar. Schließlich wäre es auch denkbar, dass sc-13179 nicht MT1 erkennt, sondern ein Protein mit Sequenzhomologien. Die Ergebnisse aus den HA-MT1-überexpremierenden BeWo Zellen lassen darauf schließen, dass MT1 ein glykosiliertes Protein mit einem Molekulargewicht von etwa 70 kDa ist. Dies unterstützt die Hypothese, dass sc-13179 entweder den glykosilierten MT1 Rezeptor nicht erkennt oder aber ein unbekanntes Protein mit Sequenzhomologien zu MT1 **Schlussfolgerung:** Es ist weitere Studien notwendig, um MT1 auf Proteinebene unter Anwendung neuer Antikörper in der Plazenta zu charakterisieren. Dies ist wichtig, da MT1 ein wichtiges Ziel für eine therapeutische Anwendung zur Behandlung von Erkrankungen in der Schwangerschaft, die mit veränderten zirkadianen Rhythmen einhergehen, darstellt.

3 INTRODUCTION

3.1 Melatonin

3.1.1 Pineal melatonin synthesis, metabolism and regulation

Melatonin (MT) or N-acetyl-5-methoxy-tryptamin has been first isolated from the bovine pineal gland [1]. It is now known as the major neurohormone secreted primarily during the dark hours at night by the pineal gland of all vertebrates, including humans. In pinealocytes, MT is synthesized in a four step enzymatic pathway [2] from the essential amino acid tryptophan (see Figure 3.1). Tryptophan is taken up from the blood and first converted by tryptophan-5-monooxygenase (T5M) to 5-hydroxytryptophan, which is then decarboxylated to serotonin (5-hydroxytryptamine) by aromatic-1-amino acid decarboxylase (AAAD). The synthesis of MT from serotonin is catalysed by the two enzymes arylalkylamine-N-acetyltransferase (NAT/AANAT) and hydroxyindole-O-methyltransferase (HIOMT), respectively. NAT acetylates serotonin to N-acetylserotonin that is finally converted by HIOMT to MT. NAT is usually the rate-limiting enzyme of MT-biosynthesis and was consequently called “timezyme” [3]. This enzyme can be regulated at different levels as outlined below. Apart from this “classical pathway” of MT formation, however, in other species and/or tissues the synthesis steps can also take place in different sequential orders, other enzymes can be involved in MT synthesis or may become the rate-limiting enzyme (for greater detail see [4]).

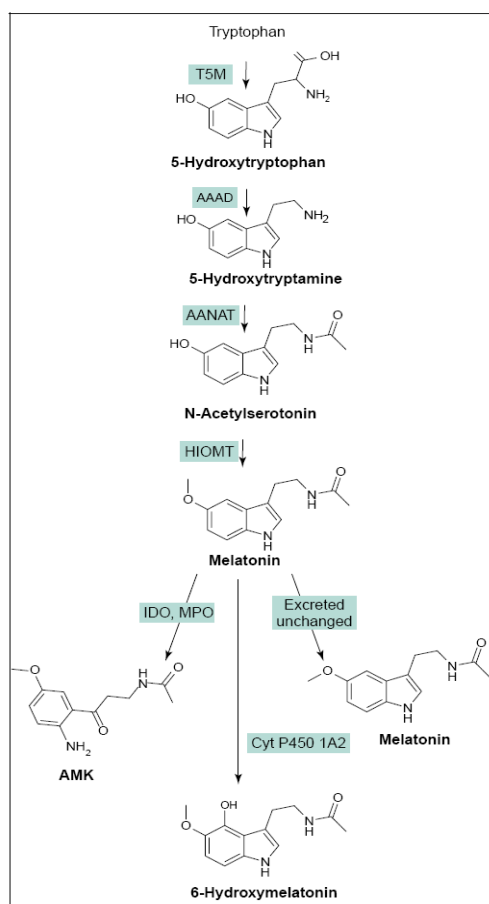


Figure 3.1 Synthesis and metabolism of melatonin

Four enzymes are responsible for converting tryptophan to MT: tryptophan-5-monooxygenase (T5M), aromatic-1-amino acid decarboxylase (AAAD), arylalkylamine-N-acetyltransferase (AANAT/NAT), and hydroxyindole-O-methyltransferase (HIOMT). MT is eliminated from the body by a classical hydroxylation pathway mediated by cytochrome P450 1A2 (Cyt P450 1A2), followed by further conjugation processes with e.g. sulphate or glucuronic acid. Alternatively, oxidation is catalysed by enzymes such as indoleamine-2, 3-dioxygenase (IDO) or myeloperoxidase (MPO) resulting in production of the unstable compound N^1 -acetyl- N^2 -formyl-5-methoxy-kynuramine (AFMK) that is de-formylated to N^1 -acetyl-5-methoxy-kynurenine (AMK). A proportion of MT is excreted unchanged (from [5]).

The pineal gland does not store MT; instead MT quickly diffuses out of the pinealocytes into the capillaries and the cerebrospinal fluid of the third ventricle [6]. This is followed by a rapid distribution to various fluids, tissues and cellular compartments such as saliva, urine, preovulatory follicle, semen, amniotic fluid and milk [7, 8]. MT displays high lipid and water solubility facilitating passage across cell membranes.

The plasma hormone profile reflects the pineal activity. The average MT profile shows an evening rise between 8.00 and 12.00 p.m., reaches peak values between 2.00 and 4.00 a.m., and then drops to low daytime levels (~10 pg / ml). Circulating nocturnal levels are usually 10 – 20 times higher than daytime concentrations. However, even among individuals of the same age, great variability in nocturnal secretion patterns and blood concentrations exist [9, 8]. Studies of twins suggest that these differences have a genetic background [10].

Metabolism of circulating MT occurs mainly by two processes (see Figure 3.1). In the liver, metabolism includes hydroxylation by cytochrome P450 monooxygenases followed by conjugation with sulphate to form 6-sulfatoxymelatonin, while glucuronide-conjugation is limited [11, 12]. Other important metabolites that are generated especially in the brain are kynuramine derivatives [13]. These derivatives are produced by oxidative pyrrole-ring cleavage. The primary cleavage product being N¹-acetyl-N²-formyl-5-methoxykynuramine (AFMK) that is proposed to be the primary active metabolite of MT. AFMK is then deformylated to N¹-acetyl-5-methoxykynuramine (AMK) either by arylamine formamidase or hemoperoxidases. Although found at low urinary concentrations, kynuramines are estimated to contribute to about one-third of the total MT catabolism [14, 15, 4]. In addition, a proportion of MT is excreted unchanged. To add to the complexity, MT (as well as kynuramines) can be metabolized nonenzymatically in all cells and also extracellularly by free radicals and some other oxidants [16]. Tissues of neuronal origin such as pineal gland and retina also contain MT-deacetylating enzymes that produce 5-methoxytryptophol (5-MT), a bioactive compound itself [17, 18, 4].

The circadian pattern of MT secretion from the pineal gland is regulated by the biological clock residing within the hypothalamic suprachiasmatic nuclei (SCN) in mammals [19]. The SCN is synchronized to the environmental light-dark cycle by light perceived by the retina. Retinal ganglion cells containing the photopigment melanopsin transfer neural messages to the SCN via the retinohypothalamic tract [20]. The SCN then regulates the function of the pineal gland through a polysynaptic network that involves the paraventricular nucleus of the hypothalamus, the medial forebrain bundle and reticular formation, as well as the intermediolateral horn cells of the spinal cord, where preganglionic sympathetic neurons innervating the superior cervical ganglion are located [21]. Postganglionic sympathetic neurons of the latter terminate in the pineal gland and release the neurotransmitter

norepinephrine (NE) during the “night” portion of the circadian pacemaker cycle provided that this occurs in a dark environment.

NE activates β 1-adrenergic receptors on pinealocytes leading to a marked rise in intracellular cAMP levels. In addition, α 1-adrenergic receptors produce a sharp increase in intracellular calcium (Ca^{++}), activation of protein kinase C (PKC) and synthesis of prostaglandin [23].

Together, these signals stimulate the synthesis of proteins, among them the MT-synthesizing enzymes, and in particular the rate-limiting NAT [25]. NAT can be regulated at different levels, that of gene expression and that of enzyme activation and stability depending on the species and the organ. In mammalian pineals, Ca^{++} /cAMP response elements (CREs) in the NAT promoter can be involved in transcriptional regulation following sympathetic stimulation. At the post translational level, NAT is regulated by phosphorylation and interaction of phosphorylated NAT with a 14-3-3 protein resulting in stabilization of the enzyme. In vertebrates phosphorylation involves PKA or PKC that are activated via cAMP or Ca^{++} . The stability of this complex, however, must remain moderate. Regular dissociation of the complex, dephosphorylation and rapid proteasomal degradation are a major component of the photic turnoff mechanism that, in addition, might be accompanied by a shutoff at the level of transcription. Upregulation of MT formation is complex and involves also a variety of neuropeptides and glutamate modulating the NE response, and several feed-back mechanisms. To add to the complexity, the various mechanisms of regulation are effective to a different extent among species [26, 4].

In summary, mammalian pinealocytes function as ‘neuroendocrine transducers’ to secrete low MT levels during daytime and high levels during night; consequently, MT is often referred to as the ‘hormone of darkness’.

3.1.2 Extrapineal melatonin

While the pineal gland of vertebrates is the best known site of MT synthesis, the production of MT is not restricted to this organ. Indeed, there is far more MT outside than inside the pineal gland and circulation, but the extrapineal functions is not well understood. With a few exceptions (see below), MT is only poorly released from “no-pineal” organs. Therefore, MT does not always act as a typical hormone that is synthesized by an organ, released into and transported by a bodily fluid to a distinct place where it acts onto the target cells via specific receptors. Depending on the physiological situation, MT can sometimes act as a hormone, but also function as a tissue factor, a paracoid, an autocoid, but also without intervention of a receptor as an anti-oxidant [27].

Extrapineal MT synthesis has been identified in the retina [28, 29, 30, 31, 32, 33], the rodent Harderian gland [34, 35], and the parietal organs of reptiles [32]. Similar to the pineal

gland, retina and parietal organ generate robust, nocturnally peaking circadian rhythms. However, with the exception of some avian and amphibian retinas, MT is poorly released from these organs and therefore might not (only) act as a hormone. Retinal MT was found to efficiently downregulate dopamine function and release [28, 30]. In the Harderian gland of rodents, a circadian rhythm of MT is almost absent.

MT biosynthesis has also been demonstrated or is assumed to occur based on the presence of the enzymes required for its biosynthesis in various other tissues and cells, but in most cases, a transmission of dark signals seems highly unlikely or impossible. The gastrointestinal tract contains 400 – 500 times more MT than the pineal gland. MT is, however, not only produced in enterochromaffin cells, but also taken up from food. Furthermore, MT is released from the gastrointestinal tract into the circulation, but also taken up from the circulation and consequently, the gut can act both as a MT source and sink [36, 37]. Other sites of MT biosynthesis include the skin [38], the bone marrow [39], lymphocytes [40], platelets and erythrocytes [41]. In addition, local MT synthesis has been suggested in tissues associated with female reproduction such as ovary [42, 43] and placenta [44, 45] as detailed below (see 3.5).

MT has also been extracted from the seeds and leaves of a number of plants and its concentration in some of these sources is several orders of magnitude higher than its night-time plasma value in humans [46]. Meanwhile, MT has been detected in all major taxa studied so far, including bacteria, dinoflagellates and other eukaryotic protists, macroalgae, plants, fungi, and various groups of invertebrate and vertebrate species [4]. It has been suggested to represent one of the first biological signals, which appeared on earth [12].

The absence of robust MT rhythms in tissues synthesizing MT suggests roles different from the transmission of dark signals. Instead, a protective function of MT might be assumed in those tissues with considerable higher MT levels than in the circulation. Indeed, a variety of anti-oxidant, but also anti-inflammatory, anti-excitatory and oncostatic effects of MT have been described and reviewed several times [47, 37, 27, 48, 41, 419, 49, 50]. Since, however, many of these effects have been demonstrated following application of high doses of MT (i.e. pharmacological doses), their physiological significance even in tissues rich in the indoleamine remains to be demonstrated (for discussion see [418]). For an exclusive role in direct radical scavenging, MT quantities might be frequently insufficient. However, tissue MT might also serve as a source for bioactive metabolites, and moreover, the anti-oxidant properties of MT are based on multiple mechanisms and MT-based effects may rather serve to avoid radical formation than to detoxify radicals already formed (see 3.1.1; [50]).

Considering the fact that MT is thought to cross any membrane because of its amphiphilicity, it is surprising that several tissues can retain MT above plasma levels [37, 48, 50]. This might be explained by sequestration of tissue MT by non-receptor binding sites

such as binding to calreticulin [51], abundant nuclear proteins [52] or yet unidentified molecules [4].

3.1.3 Functions of melatonin

A plethora of studies exist demonstrating actions of MT in almost any physio-pathological process [53, 24]. However, it should be mentioned that some of these studies are based on the use of “pharmacological” doses ($> 1 \mu\text{M}$), while others use “physiological” doses (nanomolar range). These widely diverse functions of MT are classified to be either receptor-mediated or non-receptor-mediated actions (see also Figure 3.3). Several major functions of MT in mammals are mediated by two membrane receptors, MT1 and MT2 [54, 55, 56], respectively (see 3.1.2 and 3.2.1). Furthermore, intracellular binding sites such as the enzyme quinone reductase 2 [57], nuclear receptors of the retinoic acid receptor family, e.g. $\text{ROR}\alpha 1$ and $\text{ROR}\alpha 2$ [58, 59] or (proposed) mitochondrial binding sites [60] may be involved in some aspects of MT function (e.g. protection against oxidative stress, immune modulation or inhibition of the mitochondrial permeability transition pore, respectively; (see 3.1.2 and 3.2.2, [61, 60])). Finally, although the antioxidative protection of MT is partially based on receptor-mediated mechanisms (e.g. regulation of expression of anti-oxidative enzymes, see 3.1.2), some anti-oxidant actions (e.g. direct scavenging of free radicals and electron exchange reactions with the mitochondrial respiratory chain) are receptor-independent (see 3.1.1).

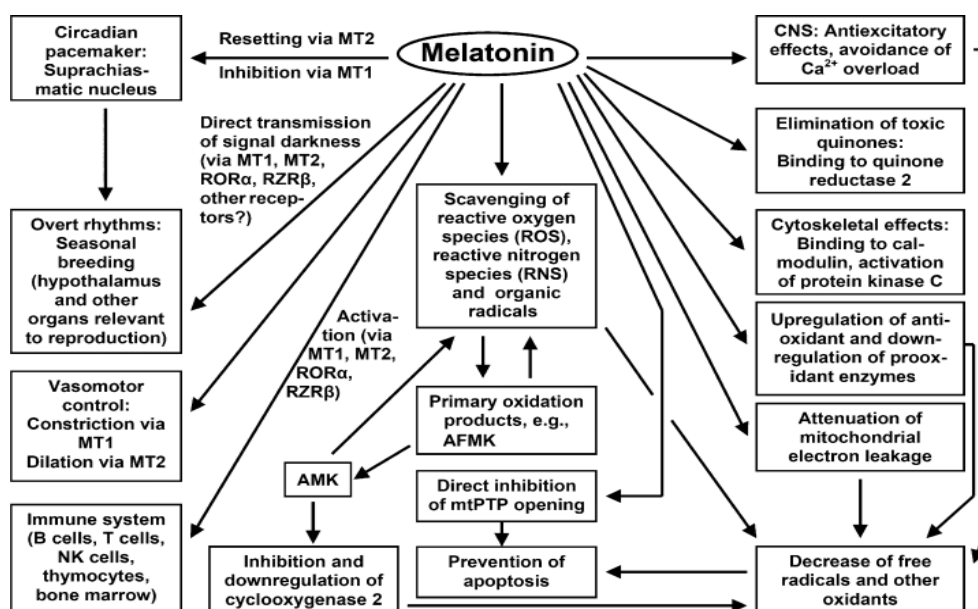


Figure 3.3 Overview of major actions of MT

MT1 and MT2, MT membrane receptors 1 and 2; mtPTP, mitochondrial permeability transition pore; $\text{ROR}\alpha$, $\text{RZR}\beta$, nuclear receptors of retinoic acid receptor superfamily; ROR, reactive oxygen species; RNS, reactive nitrogen species; AFMK, N^1 -acetyl- N^2 -formyl-5-methoxykynuramine; AMK, N^1 -acetyl-5-methoxy-kynurenine; CNS, central nervous system (from [53])

3.1.4 Non-receptor-mediated actions of melatonin

Cells and organisms containing MT in micromolar range concentrations profit from direct scavenging of hydroxyl radicals [62, 63, 64, 53]. The presence of MT in plants may help to protect them from oxidative damage and from adverse environmental insults [65]. Based on the high concentrations of MT present in some plants, it has been suggested that dietary MT derived from plants may be a good supplementary source of anti-oxidants for animals [66, 37].

During the process of radical detoxification by MT, other metabolites are formed such as AFMK and AMK that are equally effective or even better scavengers than the parental molecule [67]. In addition, at least in *in vitro* experiments, MT has been shown to synergize with other anti-oxidants (e.g. vitamins C and E) to reduce free radical damage [68, 69].

Importantly, MT, AFMK and AMK not only add to radical detoxification, but, furthermore, formation of reactive oxygen and nitrogen species can be reduced by MT and its metabolites. In this context, the capability to undergo reactions with electron exchanging and transporting systems such as the respiratory chain is of importance [48]. Certain complexes (e.g. complex I) of the electron transport chain in mitochondria seem to be particular targets of MT and may also be associated with a high-affinity binding site for MT [47]. By supporting complex I activity, MT (and metabolites such as AMK) increase mitochondrial respiration and ATP synthesis [70, 71, 72]. However, many details of the concept of mitochondrial radical avoidance remain to be resolved [48, 50]. In addition, mitochondria play an important role in the induction of apoptosis and several reports exist that demonstrate antagonisation or prevention of apoptosis by MT by modulating mitochondrial function [60, 50, 73, 74, 63].

In terms of clinical significance, a therapeutic role for the anti-oxidant actions of MT was suggested in several diseases associated with increased oxidative stress such as neurodegenerative diseases (e.g. Alzheimer's disease (AD), Parkinson's disease (PD)); [75, 76, 77]. MT has been shown effective in transgenic mouse models of AD, where it reduced oxidative stress and neuronal apoptosis [78, 79, 80], protects neuronal cells from neurotoxin-induced cell damage in *in vitro* systems of PD [76, 81] and improved sleep and circadian abnormalities in AD and PD patients [82, 83, 84].

3.1.5 Receptor-mediated actions of MT

MT is known as a **chronobiotic**, a substance that adjusts the timing or reinforces oscillations of the central biological clock [85]. In man, the 24 h blood MT cycle is synchronized with the sleep/wake cycle and the body temperature rhythm. Under normal conditions, these three rhythms have a stable phase relationship with maximal sleepiness coinciding with highest MT levels and minimal core body temperature [86]. Exogenous MT administration can alter the timing of bodily rhythms, including sleep, core body temperature

or endogenous MT [87]. The phase-shifting effects of MT are most likely mediated via MT2 receptors in the SCN [88] and can either phase-advance or phase-delay the circadian clock depending on the time of MT administration, i.e. evening/early night or late night/early morning, respectively [89].

In diurnal animals, including humans, MT promotes **sleep** [90] and seems to be involved in the physiologic regulation of sleep [91]. Presumably via MT1 receptors in the SCN, MT facilitates sleep by inhibition of the circadian wakefulness-generating mechanism [92]. There is evidence that MT can ameliorate “jet-lag” symptoms in air travellers [87, 93]. Sleep disorders, difficulties in alertness and fatigue in shift-workers and during jet-lag are based on dyssynchronization of the circadian cycles. Furthermore, circadian rhythmicity is disrupted with ageing [91, 94]. Based on a meta-analysis of [95] including 17 different studies studying sleep disorders, the use of MT was suggested in the treatment of insomnia, particularly in aged individuals with nocturnal MT deficiency [96]. Sleep disorders in children with neurodevelopmental disorders have also been treated successfully with MT [97]. There are, however, also studies arguing against a sleep promoting function of MT [98].

Altered MT levels have been shown in depressed patients [99, 100]. MT1 (-/-) mice were demonstrated to exhibit depressed like behaviour [101] and agomelatine, an MT agonist, was successfully used to **treat depressive disorders** [102, 103].

An association between MT and **bone metabolism** exists in that MT reduces bone resorption and has bone protective effects [104, 105, 106, 107]. In human bone-forming osteoblasts expressing MT receptors, MT stimulates – in a concentration dependent manner – cell proliferation and alkaline phosphatase activity [108]. Furthermore, MT might be able to direct the differentiation of progenitor cells towards osteoblasts rather than adipocytes [109]. Finally, MT also suppresses the bone-resorbing osteoclasts [110, 111].

MT is present at high concentrations in the **gastrointestinal tract**, including the bile. While it certainly displays non-receptor-mediated anti-oxidative and gastro- and intestinal-protective efficacy [112], MT can additionally function through receptors in the gastrointestinal tract. It can increase duodenal mucosal secretion of bicarbonate through MT2 receptors [113] to protect the duodenum against gastric acids; in that context, an exacerbation of duodenal ulcers in human patients is correlated with low urinary MT levels [114]. Furthermore, it can inhibit contraction of the smooth muscles of the stomach, ileum and colon [36].

MT can effect autonomic **cardiovascular regulation** [115, 116, 117] and a decrease in nocturnal serum MT levels and urinary metabolites has been reported from patients with coronary heart disease [116, 118, 9]. Since MT showed effects in reducing both systolic and diastolic blood pressure in patients with essential hypertension [119], the normalization of the circadian pacemaker function by MT has been proposed as a potential strategy for the treatment of this disease [120]. The overall effect of MT on arterial blood pressure might be

mediated centrally by mechanisms controlling the autonomic nervous system [117]. Nevertheless, the vasoregulatory actions of MT appear complex, since vasodilation is mediated via MT2 receptors, whereas signalling via MT1 receptors leads to vasoconstriction and obviously, the local balance between these receptors is different [121].

MT has been demonstrated to be an **immunomodulatory substance** in a variety of animal species and in humans [53, 122]. MT is produced by cells of the immune system e.g. leukocytes; [39, 40, 41, 123], and can also activate cells of the immune system such as T, B and NK cells [124, 40, 125]. The immuno-enhancing effect depends on the ability to enhance cytokine production e.g. IL-2, 6 and 12; [126, 124], most likely in combination with anti-oxidant and anti-apoptotic actions (see 3.1.3., [76]). The function of MT in the immune system clearly involves MT receptors e.g. on lymphocytes; [59, 125, 122, 121, 127, 128, 129]. However, more work is required to resolve the interplay of membrane and nuclear receptors in the immune system. The immunoenhancing effects of MT could find application in cancer therapy [53] or in vaccination [130]. On the other hand, MT may play a role in the pathogenesis of autoimmune diseases [131].

MT has demonstrated **oncostatic effects** against a variety of tumor cells, including human breast cancer cell lines [132, 133], ovarian carcinoma cell lines [134], endometrial carcinoma [135], prostate tumors [136] and intestinal tumors [137, 138]. However, while MT was shown to act mostly anti-carcinogenic, in some cases it may even promote tumor growth [139]. The anti-carcinogenic actions of MT are primarily attributed to its anti-oxidative and free radical scavenging activity (see 3.1.3, [140]) and oxidative stress has been demonstrated to participate in the initiation, promotion and progression of carcinogenesis [141]. However, MT mediates additional, receptor-mediated effects such as increase of glutathione levels (see below), inhibition of fatty acid uptake preventing formation of mitogenic metabolites [132] or modulation of estradiol receptor α -transcriptional activity in breast cancer cells [142]. MT has been successfully applied to improve quality of life in tumour patients [143, 144]. Furthermore, the increased incidence of breast cancer and colorectal cancer seen in nurses engaged in night shift work [145] might be related to a disturbed nocturnal circadian MT signal [132].

Apart from acting as a direct anti-oxidant, MT can influence the **levels of prooxidative enzymes** via receptors in multicellular organisms including superoxide dismutase, glutathione peroxidase and glutathione reductase, glucose-6-phosphate dehydrogenase, hemoperoxidase and γ -glutamylcysteine [146, 147, 48, 50, 49]. Furthermore, MT can inhibit lipoxygenase [50] or reduce activity of nitric oxid synthase [148]. Currently, the most reliable effect is the upregulation of glutathione peroxidase, particularly in the central nervous system, but also observed in e.g. human chorion [149], but the mechanism of upregulation requires further investigations [147]. By stimulating the enzyme gamma-glutamyl-cysteine

synthase, the rate-limiting enzyme in glutathione production, MT seems to ensure a sufficient cellular glutathione level that is a major cellular anti-oxidant itself [150].

Finally, a large variety of effects of MT on reproductive physiology in animals and human has been described that is summarized separately in (see 3.5).

3.2 Melatonin receptors and binding sites

3.2.1 Membrane receptors MT1 and MT2

3.2.1.1 Structure, expression and regulation of MT1 and MT2

Two mammalian subtypes of G protein coupled MT receptors, MT1 and MT2 (in earlier terminology called Mel_{1a} and Mel_{1b}), have been cloned and characterized from human [54, 55, 121]. A third membrane MT-receptor type, called Mel_{1c} was originally only found in non-mammalian vertebrates, i.e. bird, chicken, fishes; [151]. Meanwhile, the orphan receptor GPR50 (see 3.2.1.3) has been shown to be a mammalian ortholog [152] that might be involved in seasonality [153].

Although the human MT2 receptor has a lower affinity ($K_d = 160$ pmol/l) for ¹²⁵I-MT when compared to the human MT1 receptor ($K_d = 20 - 40$ pmol/l), the binding characteristics of the two receptors are both of high affinity [121]. Probably related to this high affinity is a very low density of MT binding sites, even in tissues most sensitive to MT [154].

MT1 and MT2 are typical G protein-coupled receptors (GPCRs) with the characteristic seven transmembrane domains (see Figure 3.3), consist of 350 and 362 amino acids (39 – 40 kDa), respectively and show high identity at the amino acid level (~55 % overall and 70 % within their transmembrane domain [121, 54, 55].

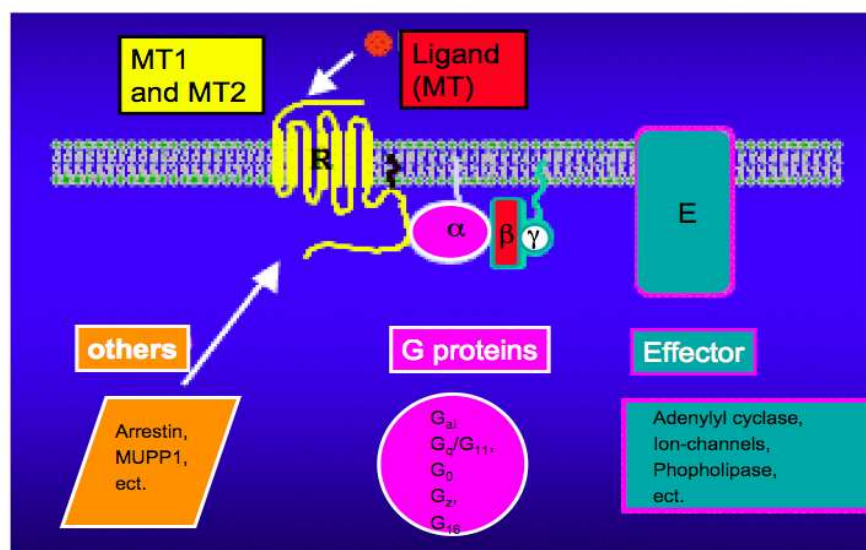


Figure 3.3 Plasma membrane receptors MT1 and MT2

MT1 and MT2 are proteins consisting of seven transmembrane domains. At the extracellular site, they interact with their ligand MT (or agonists). Intracellularly, they can interact with various G-proteins, thereby linking the MT-signal to effector molecules. Apart from G-proteins, other cytosolic interaction partners have been demonstrated, such as arrestin, MUPP1 and others

Post-translational modification sites exist, such as glycosylation sites in the N-terminal region or palmitoylatable cystein residues in the fourth intracellular loop [155]. In addition, the C-terminal domains contain putative phosphorylation sites for casein kinases 1 and 2 and protein kinases A and C [121]. Phosphorylation sites are required for internalisation, most likely via phosphorylation and β -arrestin binding; MT1 appears to be less efficiently internalised than MT2 [156]. While lipid anchor and C-terminal tail (containing the phosphorylation site) are required for G-protein interaction, their site-directed mutagenesis does not alter receptor affinity [155].

MT1 receptors are expressed in various tissues of the body including retina, other brain areas, choroids plexus, cerebral and peripheral vasculature, Harderian gland, reproductive organs, liver, kidney, gallbladder, skin, immune system and adrenal cortex [121, 157, 158]. MT2 receptors are more restrictively expressed and are found mainly in the brain, but also in other places such as lung, cardiac, aortic and coronary tissue, duodenum, adipocytes, immune cells, myometrium and granulosa cells [121]. Expression is mainly investigated at the mRNA level due to a lack of reliable antibodies especially in species other than humans [154]. Localization of human MT1 and MT2 protein has been documented in a variety of brain areas [159, 160, 161, 162, 163, 164, 165].

MT down-regulates some of its receptor population [136]. In the rodent SCN and pars tuberalis, MT1 mRNA expression and ^{125}I -melatonin binding exhibit daily variations, with

elevated levels occurring during daytime. Light exposure during the night also increases ^{125}I -melatonin binding, coincident with the suppression of MT synthesis [166, 167]. Furthermore, Estradiol appears to regulate MT binding site density and affinity and responses in the rat hypothalamus [168, 169]. Aging and Alzheimer's disease are associated with decreased MT1 and MT2 expression in the human SCN and cortex [170, 165, 171], although an increase in MT1-receptor immunoreactivity in the hippocampus of AD patients has been reported [161]. Collectively, these studies reveal the complexity of the mechanisms regulating MT receptor expression.

From the data available, it seems that in mammals all chronobiotic effects via the SCN are mediated by MT1 and MT2. MT is produced by the pineal gland under the control of the SCN. The action of MT on the SCN represents a feedback mechanism involved in the readjustment of the oscillator. MT affects phase and amplitude of the circadian oscillation in the SCN. While MT1 suppresses the neuronal firing, phase shifting is preferentially mediated via MT2 [121, 172, 173, 88]. As the two receptor types are complementary to each other, they can, to a limited extent, substitute for each other [174]. Seasonal control, especially sexual activity, in seasonal breeders is also mediated via MT1 and MT2 expressed in the median eminence and pars tuberalis. Apart from that, expression in the various other tissues mentioned, sometimes in a species-specific manner, can, but not necessarily is of chronobiotic nature.

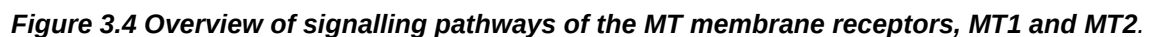
3.2.1.2 MT1 and MT2 mediated signalling involving G-proteins

The signalling mechanisms of MT have been best studied in the SCN. Signalling via MT1 and MT2 is complex (see Figure 3.4; [175, 176, 121]). Signalling through different G protein subforms has been observed, but this appears to be cell-type specific and depends on the expression of α -subunits and the availability of their downstream interaction partners in a given cell type (see Figure 3.4). The most frequently α -subunits are α_{i2} and α_{i3} , and a prominent and often observed effect mediated by MT1 and MT2 is the α_i -mediated inhibition of adenylyl cyclase resulting in a decrease of protein kinase A (PKA) activity and CREB (cAMP/ Ca^{2+} response element-binding protein) phosphorylation [177, 158, 154]. However, parallel signalling through different G-protein subforms, signalling through $\beta\gamma$ -heterodimers or even alternate signalling exist in some cases [175, 176, 4, 121].

Beside the decline in cAMP, MT2 activation can cause a rise in protein kinase C (PKC) that seems to play a role in phase shift of the circadian rhythm [173, 178, 179]. PKC activation is independent from the decrease of cAMP; instead it can be due to activation of phospholipase C (PLC) isoforms (β or η) via α_i - or α_q - or even $\beta\gamma$ -G protein subunits [180]. Finally, PKC might be activated via G-protein dependent opening of ion channels [56]. In addition, inward rectifier K^+ channel (Kir channels), but also Ca^{2+} channels might be activated by MT1

presumably via $\beta\gamma$ -G protein subunits [181]; it is currently assumed that suppression of neuronal firing in the SCN by MT is mediated via these ion-channels. Furthermore, both MT1 and MT2 can interact with and signal through various pertussis-toxin-sensitive and insensitive G proteins, such as G_q/G_{11} , G_o , G_z and G_{16} , but many details of the mechanisms await their characterization [4, 177, 158, 182, 183, 184, 185].

Downstream effects, following G-protein activation via MT1 or 2 receptors are likewise multiple and complex (see Figure 3.4). As demonstrated in the vascular system, signalling pathways originating from MT1 and MT2 can even turn out to be antagonistic, in that MT1 activation causes vasoconstriction, while MT2 activation results in vasodilation [4, 121]. Following activation of PLC and PKC or release of $\beta\gamma$ -G protein subunits from G_i , the MAP kinase pathway, including MEK1/2, ERK1/2, and JNK, is stimulated and this might include additional effects via phosphoinositide 3-kinase (PI3K) and its downstream elements [186] f. On the other hand, ERK can also be stimulated via β -arrestin bound to a phosphorylated MT receptor [187]. PLC β can have other consequences such as activation of Calmodulin (CaM) kinases or the opening of Ca-activated K⁺ channels. As suggested by additional interactions of PI3K with JNK or stimulation by $\beta\gamma$ -G protein subunits [4, 183, 185], there is a cell-type specific complexity in the pathways that definitely asks for further research to understand the signalling pathways *in vivo*.



MT signalling also affects cGMP levels. However, both rises in cGMP [158] as well as MT2-mediated decreases in cGMP levels have been described [188] and in both cases the mechanisms are not completely determined.

Heterodimerization between MT1 and MT2 has been observed (see Figure 3.5), as well as homodimerization of MT1 and, to a lesser extent, MT2 [189]. Although these could be artefacts based on overexpression of the receptors in transfected cells, homo- and heterodimerization, and also oligomerization are common phenomena with respect to GPCRs. GPCR like the GABA β and various taste receptors require heterodimerization for

their functionality. With respect to MT1 and MT2, the necessity and consequence of such interactions are almost completely unknown. Numerous GPCR are still orphan receptors that exert regulatory effects on other GPCRs. Likewise, GPR50 has been demonstrated to heterodimerize with MT1, thereby abolishing high-affinity agonist binding, G-protein coupling and β -arrestin binding due to the long C-tail [190]. GPCR dimerization may have a profound impact on receptor pharmacology, signalling and regulation. Furthermore, modifications of the MT1/MT2 ratio are likely to influence the dimerization pattern. As such modified ratios have been observed in the brain and retina of AD patients [165]; they may modify the functional MT response.

MT1, but not MT2, has been demonstrated to interact with PDZ (PSD-95/Drosophila disc large/ZO-1 homology) domain-containing proteins. One of these is MUPP1 (multi-PDZ domain protein 1; see Figure 3.5). While binding of the MT1 c-terminus to the PDZ domain 10 in MUPP1 is not involved in the trafficking of MT1, the interaction seems to be important for high affinity binding of MT1 to G_i and coupling of MT1 to the cAMP pathway [191]. Another PDZ-domain containing protein is neuronal NO synthase (nNOS), although the functional consequences of this interaction are not yet known [192].

Finally, MT1 and MT2 interact with a variety of other cytoplasmic proteins reviewed recently by [154], including the actin-binding protein filamin A and insulin receptor substrate 4 (IRS4). Specific interaction-partners of MT1 are e.g. Rac1 and Rap-1A, while MT2 was found to interact with e.g. p120 catenin. In most cases, the specific consequences of these interactions need to be demonstrated.

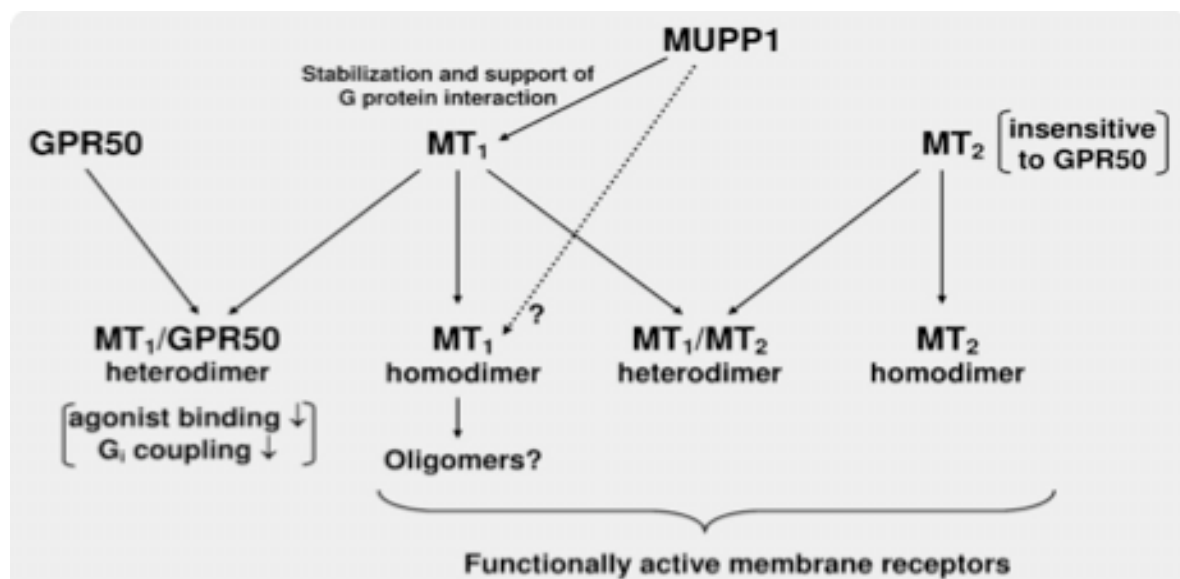


Figure 3.5 Dimerization of MT membrane receptors (MT1 and MT2) and interaction with GPR50 and MUPP1 (multi-PDZ domain protein 1; from [56])

3.2.2 Other MT binding sites

A third melatonin binding site initially suspected to be another membrane receptor and called the “MT3”, was later characterized as the cytosolic enzyme quinone reductase 2 (QR2; [57] f; [193]. QR2 has a low affinity for MT (5 – 50 nM; [194]). QR2 is expressed in brain, liver, kidney, heart, lung, intestine, muscle and brown adipose tissue [195], various synthetic ligands have been developed for this binding site and several effects have been described [4, 47]. However, the precise role of the enzyme remains to be established. Some of its polymorphic subforms have been related to Parkinson’s disease [196], while the disruption of the QR2 gene leads to bone marrow myeloid hyperplasia [197]. QR2 might be an activating enzyme enhancing the toxicity of certain substances. MT that is known to have anti-oxidant and protective effects might inhibit QR2 activity following binding [154].

MT is also a ligand for transcription factors belonging to the retinoic acid receptor superfamily (RZR/ROR), which includes three subtypes (α , β , γ) and four splicing variants of the α -subtype [198, 199, 419, 58, 59]. Their affinity for MT is in the lower nanomolar range and a synthetic ligand (CGP 52608) is often used to identify effects mediated by these nuclear proteins. However, in most studies, only expression of RZR/ROR has been demonstrated, and only few studies give information on up- or downregulation of individual genes. RZR β has been found expressed in the central nervous system, including the pineal gland. ROR α seems to be involved in some aspects of immune modulation as well as upregulation of anti-oxidant enzymes; hypoxia-inducible factor 1 α (that might also be involved in anti-oxidant action) or the clock gene *bmal1* that plays an important role in the cellular circadian oscillator [56, 419, 147, 200, 201]. Definitely, more investigations are required to clarify the physiologic functions of these MT receptors.

In addition, MT interacts with intracellular proteins such as calmodulin [202], calreticulin [51] or tubulin [203, 204] and antagonizes the binding of Ca⁺⁺ to calmodulin [205]. These interactions are most likely related to some of the physiological effects of MT but critical data regarding this point have yet to be obtained.

Finally, two mitochondrial binding sites have been identified. These might be associated with the effect of MT to prevent apoptosis and to participate in the prevention of electron leakage in the electron transport chain [56].

3.3 The placenta

The placenta is a temporary, pregnancy-associated organ. In general, a placenta forms at a contact side of the two membranes surrounding the fetus - i.e. fetal facing amnion and maternal facing chorion – and the maternal endometrium of the uterus (Figure 3.6 A). However, there is no other organ with a higher degree of species differences than the placenta [206].

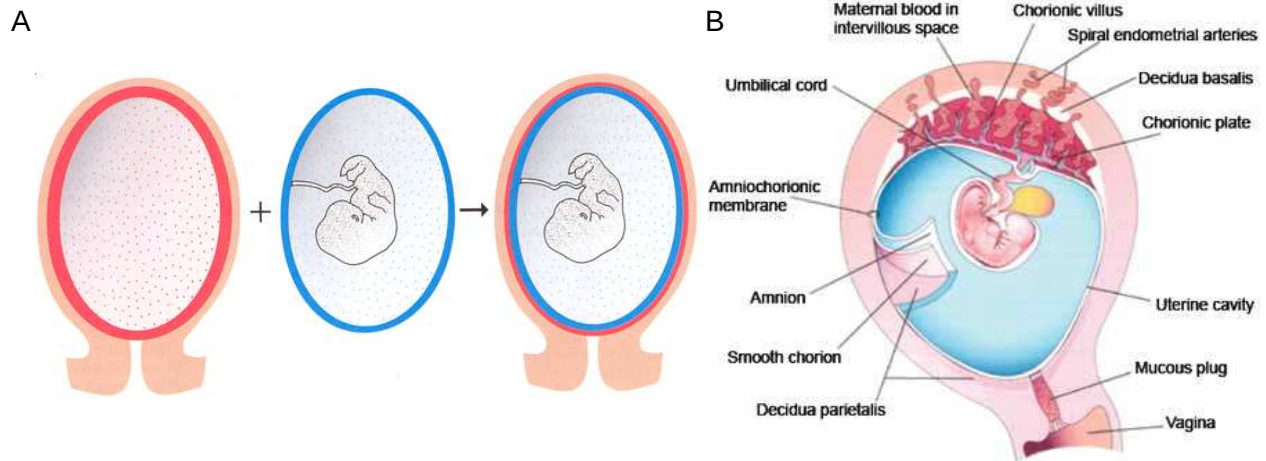


Figure 3.6 (A) A placenta is formed; when the fetal membranes (i.e. amnion and chorion) (blue) come into close contact with the endometrial lining (red) of the uterus, from: [206]. (B) Schematic depiction of the discoidal human placenta

The human placenta (Figure 3.6 B) is an *allantochoorial* placenta, where the main fetal exchange membrane is the chorion. The chorion is derived from fusion of the trophoblast from the blastocyst wall with the fetal mesenchyme; it becomes fetally vascularized from the allantois, the embryonic bladder. The amnion remains unvascularized and never replaces the chorion. It serves as an additional, inner membrane that separates the chorion from the amniotic fluid. The human placenta represents a single disk-like zone of intimate maternofetal contact (*discoidal placenta*) and it is a *villous type of placenta*, where a large exchange surface is provided by a tree-like branching pattern of the chorion (i.e. the placental villous tree). Finally, the human placenta is a *hemochorial placenta*. This refers to the structure and composition of the “placental” or “maternofetal barrier”, and defines that the (fetal) trophoblast has invaded the endometrium and destructed the uterine epithelium, the endometrial connective tissue and eventually the maternal vessels. Consequently, the trophoblastic surface directly faces the maternal blood [206].

3.3.1 Structure of the human term placenta

The human placenta and the uterus build a unit that is composed of fetal tissue derived from the chorion and maternal tissue derived from the decidua (i.e. endometrium during pregnancy). The term placenta is a local, disk-like thickening of the chorionic sac (Figure 3.7

A). It is divided into two separate sheets: the basal plate (decidua basalis), where fetal and maternal (decidual) cells are in close association, and the chorionic plate (Figure 3.7 B). At the placental margins, chorionic plate and basal plate fuse with each other, forming the chorion leave.

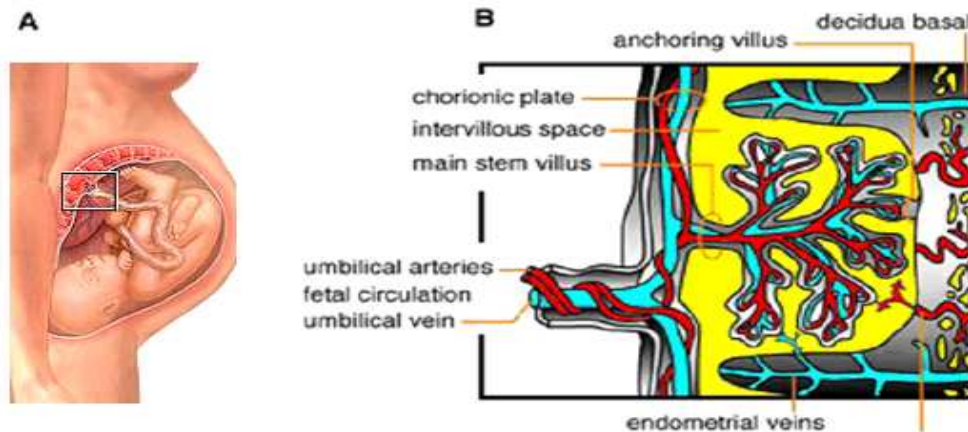


Figure 3.7 (A) The human uterus at a term stage of pregnancy. The fetus is completely surrounded by amnion and chorion leave and is connected via the umbilical cord with the discoidal placenta. The insert is shown enlarged in B **(B)** The placenta consists of a fetal side (containing fetal-derived cells), the chorionic plate, from where the villous trees as well as the umbilical cord emerge. The maternal side of the placenta (containing mainly maternal-derived cells) is formed by the decidua basalis. The villi that are anchored in the decidua basalis are surrounded by the intervillous space (yellow) floated by maternal blood. Maternal blood enters the intervillous space with high pressure via the endometrial arteries (red), floats around the villous trees and is then forced back into the maternal circulation via the endometrial veins (blue). Oxygen- and nutrient-rich blood reaches the fetal circulation via the umbilical vein (blue), oxygen-poor blood containing fetal waste products is returned via two umbilical arteries (red). from: [207].

The chorionic plate gives rise to the chorionic villi as well as the umbilical cord (Figure 3.7 B). In principle, the chorionic villi represent the functional units of the placenta. Together, the chorionic plate and the basal plate enclose the intervillous space. This intervillous space is perfused with maternal blood that enters with high pressure at the basal plate via the endometrial arteries (spiral arteries), floats around the villous trees and is then forced back into the maternal circulation via the endometrial veins.

The fetal circulatory system extends into the chorionic villi (Figure 3.7 B): Fetal blood enters the placenta via two umbilical arteries, is distributed into fetal vessels in the villi and is then returned into the fetal circulation via the umbilical vein [206].

3.3.2 Basic structure of the (terminal) villous trees

The villous trees, also called chorionic villi, are the main functional units of the placenta. They are composed of a vascular and stromal core that is covered by a mantle of trophoblast cells; all cells within the chorionic villi are derived from the fetus (see Figure 3.8).

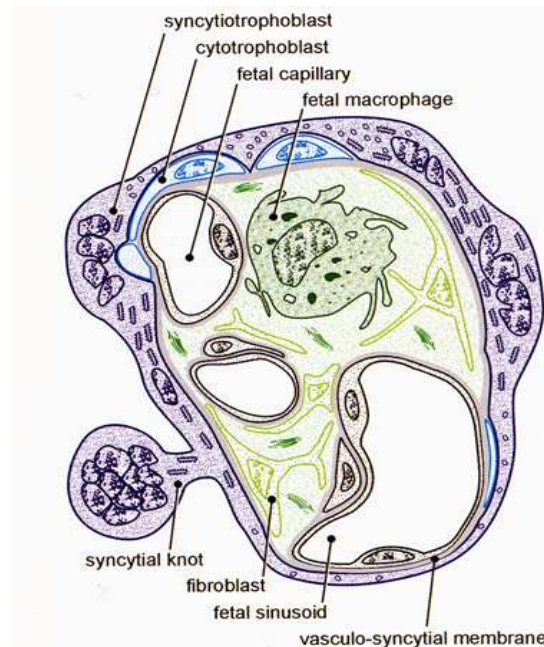


Figure 3.8 Cross section of a terminal chorionic villous showing the various cell types present in these structures, i.e. syncytiotrophoblast, cytotrophoblast, macrophage, fibroblast, endothelial cell in the fetal capillary) as well as a vasculo-syncytial membrane area. from: [206].

The villous core contains fetal capillaries, mesenchymal tissue (=stroma containing reticulum cells, fibroblasts, myofibroblasts, smooth muscle cells) and placental immune cells (Hofbauer cells). Fetal blood vessels are built from a continuous endothelium that is anchored to its basal lamina.

The trophoblast (epithelial cells) consists of an inner layer of mononucleated cytotrophoblasts (CTB) that continuously multiply, differentiate and fuse to form the uninterrupted, outer layer of the syncytiotrophoblast (STB). In the first trimester of pregnancy, the CTB form an almost complete layer of cuboidal phenotype. Later in gestation, the CTB transform to flattened cells with multiple interconnecting processes covering only about 44 % of the basal lamina [208]. The STB layer constitutes the inner surface of the placenta, faces the intervillous space and is in direct contact with maternal blood. A trophoblastic basement membrane separates the trophoblast from the underlying connective tissue stroma and forms a supportive matrix.

The *placental barrier* separates maternal from fetal blood. In the mature placenta it consists of the STB and fetal endothelial cells and the interjacent mesenchyme. In areas of high

transport capacity (mainly in the terminal branches of the chorionic villi), the mesenchyme is reduced. Trophoblast and endothelial cells are in direct contact, separated only by their basal laminae. Such areas are termed vasculo-syncytial membranes [206].

3.3.3 Functions of the human placenta

The placenta can be regarded as the first fetal organ and serves a variety of important functions.

3.3.3.1 Functions of the early placenta

During early gestation, the placenta mediates implantation of the embryo in the uterus by controlled trophoblast invasion [209, 210]. Placental production of factors such as cytokeratin, hormones and specific surface receptors ensures maternal recognition of pregnancy and helps to control implantation. In a normal uterine pregnancy, implantation is a well-balanced result of trophoblast invasiveness and decidual defense mechanisms involving not only fetal-derived trophoblast cells but also a variety of maternal cells (e.g. uterine natural killer cells, macrophages, cells of uteroplacental artery walls, [206]).

Normally, the fetal trophoblasts invade the uterine wall and consecutively the maternal spiral arteries for remodelling. An invading type of CTB, extravillous trophoblasts, occludes the maternal spiral arteries right after implantation in order to limit maternal blood flow into the placenta. In this early phase, cells are differentiating in a low oxygen environment. In this way, the developing embryo is protected from damage resulting from reactive oxygen species (ROS). Intraplacental oxygen level rises increasingly after embryogenesis, when the maternal intervillous circulation becomes fully established. If trophoblast invasion is not sufficient to occlude the spiral arteries, it comes to premature onset of the maternal intervillous circulation. Deficient trophoblast invasion during early gestation is therefore associated with increased oxidative stress. This causes extensive oxidative damage to trophoblast cells and is likely a major contributory factor to miscarriage. Depending on the extent of impairment of trophoblast invasion, placental blood flow becomes disregulated, resulting in varying degrees of placental oxidative stress, which plays an important role in the pathophysiology of preeclampsia PE; [211, 212].

3.3.3.2 Functions of the term placenta

The placenta forms an immune interface between the mother and the fetal allograft. Interactions between maternal immune cells and fetal trophoblast cells of the placenta that are semi-allogeneic would normally trigger rejection of the fetus by maternal immune response. However, during gestation several mechanisms exist that act to keep the maternal immune system tolerant of the fetus. The trophoblast plays a central role in maintaining this tolerance [213].

Along with fetal membranes and amniotic fluid, the term placenta supports normal growth and development of the fetus. The placenta provides the fetus with oxygen, water and minerals [214, 215, 216] and nutrients such as carbohydrates [217, 218], lipids [219] and vitamins [220]. Furthermore, carbon dioxide and other waste products are removed from the fetal circulation [216]. In addition, the placenta has own requirements and metabolizes a number of substances and releases metabolic products into the maternal and/or fetal circulation [221, 222, 223].

The communication between mother, placenta and fetus is ensured via blood-borne substances since the placenta is devoid of nerves. In this context, the placenta secretes various hormones (e.g. human choriongonadotropin (hCG)), growth factors, cytokines and chemokines. [216, 224]

Finally, the placenta protects the fetus against many, though not all infections and maternal diseases [225, 226, 227, 228] and xenobiotic molecules [229, 230] and by receptor-mediated transport of IgG antibodies from mother to fetus, the human placenta provides passive immunity to the newborn [207, 231, 232].

In summary, the placenta is the main interface between fetus and mother protecting the fetus and regulating intrauterine development in two ways: First, it supplies the fetus with oxygen and nutrients required for growth and energy metabolism. Second, it produces and metabolizes a range of secretory factors which affect fetal growth and development. These factors act both directly on the fetal tissues, and indirectly by metabolic actions in mother and placenta to adjust the fetal nutrient supply.

Systems to study expression and function of molecules in human term placental villi or (villous) trophoblast cells located at the materno-fetal interface

3.3.4 The placenta in situ and the in vitro perfused placenta

For immunolocalisation of molecules in situ, small pieces of placentas are processed immediately after delivery for microscopy or electron microscopy [233].

To study transport from the maternal circulation into the fetal circulation or vice versa, the *in vitro* perfused placenta most closely mimics the *in vivo* condition, because it maintains the complexity of the intact organ. The placental perfusion is not easily accomplished due to the sophisticated surgical procedure and instrumental set-up required; nevertheless, the *in vitro* perfused placenta provides a powerful system to investigate transfer of molecules across the placenta [234, 235, 236, 237].

3.3.5 Placental villous explants cultured in vitro

Small pieces of placental tissue can be cultured as tissue explants in long- or short-term cultures [238]. This system preserves the integrity of the tissue, requires only little material

and does not require elaborate techniques. It is suitable to study a variety of placental functions such as the secretion and effects of hormones [239], STB regeneration [238], expression of markers in response to certain stimuli [240] and differences in placentas derived from either healthy or pathologic (e.g. PE) pregnancies [241] or placentas of different gestational age [242].

3.3.6 Isolated, in vitro differentiated and cultured STB

Primarily CTBs are isolated and cultured for 48 – 72 h *in vitro*. During this period, they aggregate and form syncytia [243]. Since these *in vitro* differentiated cells (STB) do no longer divide, it is difficult - though not impossible - to obtain a continuous layer of polarized STB on filters [244]. This hampers studies on polarized transport of nutrients and other compounds significantly. Another disadvantage is the laborious and reagent-intensive isolation. In addition cultures are often contaminated by other placental cell types and, as a consequence, the reproducibility of the experiments depends strongly on the purity of the preparations. The major advantage of the STB cultures is that transport processes can be studied without interference of the endothelial layer [207, 234].

3.3.7 Immortalized cell lines of placental origin - Choriocarcinoma cell lines

A variety of immortalized trophoblast cell lines have been created by spontaneous transformation or by transfection, but many of them are either difficult to culture or to characterize [245, 246, 247].

Thus, choriocarcinoma cell lines are more commonly used. Choriocarcinoma are malignant tumors of epithelial (trophoblast) origin that have been shown to display characteristics of invasive trophoblasts [248]. They bear morphological resemblance to their cell of origin, the trophoblast of the healthy first trimester placenta. Therefore, choriocarcinoma cells may serve as a valid and convenient *in vitro* model system for studying cellular activities of trophoblasts and regulation of transplacental transport and uptake mechanisms.

To date, mainly three choriocarcinoma cell lines - BeWo, JAR and JEG3 cells - with different characteristics have been used [234].

JAR cells share many of the characteristics of early placental trophoblasts, such as synthesis of hCG and steroids [249], and the ability to differentiate into STB-like cells *in vitro* [248]. JEG3 cells, originally derived from the BeWo cell line [250, 251], express abundant hCG and placental lactogen [251]. They form large, multinucleated syncytia in culture [252], which resembles that of STB *in vivo*. The BeWo cell line is comprised of CTBs with no differentiation to syncytium under non-activated conditions [253, 254]. This is in contrast to primary cultures of term CTBs, which aggregate and form syncytia (see 3.3.6). However, the BeWo cell line so far is the only choriocarcinoma cell line that can be cultured as a tight

polarized monolayer on permeable filters, where it develops apical and basolateral membrane domains and domain-specific expression of proteins [255, 256]. Consequently, it can be used to study interaction of molecules with the apical (maternal) surface and transport across the STB layer [257, 258, 259, 260, 261, 262, 263].

3.4 Preeclampsia (PE)

3.4.1 Definition of PE

Preeclampsia (PE) is a multisystem disorder of the human pregnancy, affecting between 2 and 7 % of all pregnancies in the Western world. In developing countries, the incidence can be even higher. PE is a major cause of maternal and fetal morbidity and mortality, responsible for 18 % of all maternal deaths worldwide.

PE is defined by the presence of new onset of hypertension (a blood pressure of ≥ 140 mmHg systolic or ≥ 90 mmHg diastolic) associated with proteinuria (≥ 0.3 g protein in a 24 h urine sample) after 20 weeks of gestation [264, 265]. Besides hypertension and proteinuria, PE can cause a number of other clinical symptoms (e.g. edema, thrombocytopenia) by affecting multiple maternal organs. PE is regarded as serious, if severe hypertension (≥ 160 mmHg systolic or ≥ 110 mmHg diastolic) combines with proteinuria or hypertension combines with severe proteinuria (≥ 5 g protein in a 24 h urine sample) or if multiorgan involvement is observed (e.g. seizures, oliguria, thrombocytopenia, abnormal liver enzymes, persistent and severe CNS symptoms).

A fraction of PE women (5 – 8 %) develops the so-called HELLP syndrome - a severe variant of PE - presenting with hemolysis, elevated liver enzymes, and low platelet counts. Moreover, PE can progress to eclampsia, which is defined as the onset of convulsions in women with either gestational hypertension or PE. It represents an extremely dangerous state for mother and fetus [266, 265, 264, 267, 268]. Women who suffered from PE have a higher risk for health disorders later in life, such as an increased risk for cardiovascular diseases [267, 266, 269, 270, 271].

In addition, preterm delivery, intrauterine growth restriction (IUGR) and perinatal death are associated with PE. Babies born prematurely, with low birth weight, or after exposure to a stressful environment - such as during PE - are at risk to suffer from short- and long-term complications [272, 265].

3.4.2 Pathogenesis of PE

3.4.2.1 PE – the „disease of hypotheses“

PE has been called the “disease of theories and hypotheses” [273], since various hypotheses have been proposed over the years regarding its pathogenesis, indicating the complexity of this disorder. Based on the detection of new and early biomarkers for PE

[274, 275] in the recent years there has been a shift in defining the origin of PE from midgestation towards very early stages of pregnancy. The pathogenesis of PE is likely to be multifactorial [264, 265, 276, 277, 278].

3.4.2.2 The role of maternal endothelial dysfunction

Maternal endothelial dysfunction is linked to the pathophysiology of PE [265, 277, 278], including inappropriate endothelial-cell activation, enhanced endothelial-cell permeability and platelet aggregation. An endothelial defect in the kidney is likely to be the cause of proteinuria. Failure of endothelium-dependent vessel dilation contributes to the development of hypertension. Furthermore, intense vasoconstriction in different organs leads to hypoperfusion. The increased small vessel permeability can contribute to the development of cerebral edema and increased CNS (central nervous system) permeability. In addition, vascular thrombosis plays an important role in the pathophysiology of the HELLP syndrome [268, 279]. Women with pre-existing disorders – including diabetes, obesity and essential hypertension - are predisposed to PE. Endothelial dysfunction is common to all of these disorders, thus adding support for a central role of the endothelium in the disease process [280].

Importantly, the fetus does not develop clinical symptoms similar to the maternal syndrome. Instead, fetal morbidity and mortality are believed to result exclusively from placental insufficiency caused by impaired uteroplacental blood flow or placental infarction [265].

3.4.2.3 The role of the placenta

The placenta, but not the fetus is essential for development and maintenance of PE, since PE has also been observed in patients with molar pregnancy where a fetus is absent. Delivery is the only cure, but the disease may persist if remnants of placental tissue are retained [266, 272, 265, 277]. The placenta of women with PE is abnormally structured, with histological evidence of vasculitis, thrombosis, and areas of ischemic or necrotic tissue. In addition, these placentas exhibit increased trophoblast apoptosis. These combined observations have led to the speculation that maternal endothelial dysfunction, as the key feature of PE, is related to circulating factors produced by the abnormal placenta [264, 266, 272, 265, 277, 281].

3.4.2.4 Deficient utero-placental blood flow

During the normal implantation and placentation process, extravillous CTB invade the decidua and the myometrium in order to remodel the uterine spiral arteries. The trophoblast cells transform the maternal spiral arteries from small caliber resistance vessels to large

caliber capacitance vessels. These changes result in vasodilation and a decrease of vascular resistance and provide an adequate blood flow to the fetus.

Aberrant placentation and an “utero-placental blood flow deficiency” are claimed to play a central role in the pathophysiology of PE. Two pathways eventually result in deficient utero-placental blood flow and can also be found combined: the first is an insufficient trophoblast invasion that fails to remodel maternal spiral arteries leaving them narrow. This is referred to as the “fetal pathway” of PE development. In the so-called “maternal pathway”, the spiral arteries are blocked due to thrombosis and atherosclerosis raised by maternal disorders [282].

In any way, the consequence is a reduced perfusion of the placenta, due to relative vasoconstriction of the feto-placental vessels. Persistent hypoxia (or intervals of hypoxia followed by reoxygenation) of the placenta in PE result in increased activity of xanthine oxidase in trophoblasts to generate reactive oxygen species (ROS) thus leading to oxidative stress. ROS are released from the placenta into the maternal circulation and contribute to maternal endothelial dysfunction [264, 265, 281]. Hypoxic damage of the villous trophoblast enhances apoptosis and as a consequence, placental debris is released into the maternal blood stream. Hypoxia may also stimulate the release of other factors from the trophoblast, such as TNF- α [265, 281]. In summary, aberrant placentation might contribute to the pathogenesis of PE by triggering an increased systemic maternal inflammatory response with activation of macrophages, a raised production of proinflammatory cytokines and an imbalance of angiogenic factors causing the widespread endothelial dysfunction, which characterizes PE [265]. However, this concept of failure in remodeling of the spiral arteries as a major cause of PE has recently been questioned [274].

3.4.2.5 Placental derived factors causing endothelial dysfunction

Following the hypothesis that placental underperfusion represents the ‘first stage’ in PE, research has aimed to identify placental factors, which are produced in response to the hypoxic state and are subsequently released to the maternal circulation. There is quite profound evidence for oxidative stress to play a central role in the development of endothelial dysfunction in PE [264, 283, 284].

However, a number of other factors have been found that are supposedly detrimental to the endothelium by acting either directly or indirectly. These factors include trophoblast microparticles [285, 283], inflammatory mediators such as TNF- α , IL-6 and IL-1 [281, 276], factors of the renin-angiotensin system [286, 287] and angiogenic factors (e.g. placental soluble fms-like tyrosine kinase 1 (sFlt1); [288, 289] and soluble endoglin (sEng) [289].

Several risk factors have been identified for PE, such as previous history of PE and maternal co-morbidities, including obesity, insulin resistance, diabetes, chronic hypertension, and others [265, 280]. In addition, genetic factors appear to contribute to the development of

PE (in association with environmental factors), but their precise role remains unclear [264, 290, 291, 292, 293].

3.4.3 Treatment of PE

The treatment of PE has not changed significantly over the past decades. PE is still treated symptomatically (e.g. antihypertensive treatment) with emphasis on prenatal care, early diagnosis and timely delivery [264, 265]. The benefit of various methods that are currently used to prevent PE (e.g. aspirin, heparin, anti-oxidant supplementation) remains unclear [294].

3.4.4 Conclusion

Placental oxidative stress plays a central role in the pathogenesis of PE. At present, no reliable predictive test tool exists for identifying pregnant women who are going to develop PE. Because PE has a high impact on maternal as well as neonatal morbidity and mortality, it continues to be a subject of intensive research aimed at identifying all facets of the multifactorial pathogenesis, new and effective serum markers for risk assessment and new possibilities to treat the disease.

3.5 Melatonin and reproduction

MT is suggested to participate in a variety of reproductive processes; the best investigated being the synchronization of the reproductive response to appropriate environmental conditions in photoperiodic animals (see 3.5.1; [81, 295, 296]).

MT can cross the placenta during pregnancy and mediates important photoperiodic information to the fetus [295].

Anti-oxidative systems and molecules are of importance in all cells and organs, but especially in the reproductive system. Although free radicals are key signalling molecules for various reproductive functions in e.g. oocytes, sperm and follicular fluid [297], changes in these microenvironments can directly influence follicular development, ovulation, quality of oocytes, sperm-oocyte interaction, implantation, and early embryonic development (see 3.5.3. and 3.5.4; [298]). Various pathologies affecting the female reproductive tract such as endometriosis, ovarian cancer or polycystic ovary disease, but also pregnancy-related diseases such as PE (see 3.5.4) might be the result of an excess of free radicals and there is recent evidence for MT having potential roles in the pathophysiology of these diseases [298].

MT might exert its function via different mechanisms (direct or indirect) and at multiple levels of the reproductive system [295, 296] as MT receptors have been demonstrated in hypothalamic neurons governing the release of pituitary gonadotrophins [299, 121], in gonadotropes of the anterior pituitary [300, 301, 302], in female and male gonads [303, 304],

in the myometrium [305] and in the placenta [45, 44]. In most cases, very little is known on the consequences of MT-receptor interaction in these tissues, and definitely more research is required to understand all functions MT exerts in the reproductive system. In the human placenta, expression of MT receptors has been described only recently [44] and functional studies are still lacking.

3.5.1 Melatonin and seasonal reproduction

The **reproductive competence** in photoperiodic species, such as sheep, mink, ferret, skunk, horse, hamster and feral mice, depends on seasonally-changing day lengths. Proportional to the daily dark period, the pineal gland determines the seasonal reproductive cycle via elevated nocturnal secretion of MT [306, 307, 308, 81, 295]. Long-day breeding animals (many rodents) are sexually depressed during the winter months, their reproductive regression being associated with an extended period of MT elevation. In contrast, short-day breeders such as sheep are sexually most active during those days of the year. These originally contradictory findings finally led to the understanding that MT is neither anti-gonadotrophic nor pro-gonadotrophic *per se*, but provides the reproductive system with calendar information [309, 310, 311]. This information in combination with the species-specific gestational period adjusts the testicular and gonadal function in a way that guarantees birth of the young in the spring and thereby their highest survival rate. The way MT modulates reproductive function is not completely clear today. Definitely, the medial basal hypothalamus and the anterior pituitary gland contain MT receptors [312, 313, 314, 315, 121]. MT has been shown to down-regulate gonadotropin-releasing hormone (GnRH) in a cyclical pattern; this inhibition involves various MT receptors (MT1, MT2, ROR α and RZR β). In the pituitary gland of mice, where MT1 and MT2 receptors are expressed, MT inhibits GnRH-induced Ca²⁺ signalling and gonadotrophin secretion [299, 301, 316, 191, 317, 318, 319, 320]. MT has also been shown to have a direct effect on the female reproductive tract, where it regulates sex steroid secretion in hamster [321] and human [303, 322]. Although these cellular and molecular mechanism are only beginning to be understood [323, 296], MT has meanwhile been successfully applied as a pharmacological agent to influence the breeding season of sheep and increase lambing [324, 325].

In humans, a suppression of the reproductive function by MT and the originally suggested function as a contraceptive [326] has not been confirmed. Nevertheless, there appears to be a relationship between high MT concentrations and hypothalamic-pituitary-gonadal hypofunction [327]. Although humans are not seasonal breeders, seasonal changes in reproductive performance do occur, probably involving MT secretion [328].

3.5.2 Melatonin and puberty

A causal relationship between the onset of human puberty and a decrease in pineal MT production occurring at this developmental stage has originally been suggested [329, 330, 296]. This hypothesis was supported by the observations of low MT levels in cases of precocious puberty and decreased MT levels after successful treatment of delayed puberty with gonadotropin releasing hormone [331]. Later reports, however, could not correlate MT and puberty [332, 333], and a recent longitudinal study rejected the hypothesis of a key role of MT in initiation or progression of human pubertal development [334]. The originally observed association of MT with pubertal development may be the product of maturation of the neuroendocrine–gonadal axis, rather than reflecting a regulatory role of MT as suggested by [335].

3.5.3 Melatonin, ovulation and fertility

There is strong recent evidence that MT protects human oocytes from free radical damage [295]. MT concentrations in human ovarian follicular fluid derived from the antra of Graafian follicles were found higher than corresponding plasma concentrations [336, 322], indicating either concentration of serum-derived MT against a gradient or synthesis in ovarian tissue that was found to express the relevant enzymes NAT and HIOMT [43]. From the follicular fluid, MT seems to diffuse into the cumulus oophorus and oocytes and protects them from free radical damage as investigated in human oocytes retrieved for *in vitro* fertilization and embryo transfer [295]. Exogenous application of MT or Vitamin E to women prior to oocyte retrieval significantly increased intrafollicular MT concentrations and reduced DNA and lipid damage, both markers for oxidative stress [295]. In comparison to untreated women, exogenous application of MT doubled the fertilization and pregnancy rates in women undergoing *in vitro* fertilization and embryo transfer. Likewise, MT seems to protect mouse [337, 295], buffalo [338], cow [339] and pig [340] oocytes from oxidative stress. In summary, MT has a positive impact on processes related to ovulation and early embryo development. It remains to be determined, whether this is accomplished only by its direct free radical scavenging properties, or also by action via MT receptors that were documented on some ovarian cells [338].

There is also evidence for protection against free radicals of male sperms during spermatogenesis [341, 342] and reduction of the level of oxidative stress in testis [343]. Furthermore, human seminal fluid contains MT and it was observed for hamster sperms, that MT positively influences hyperactivation, the specialized movement of the flagellum of sperms, permitting the penetration of the zona pellucidum. This is mediated via MT1 receptors [344]. In contrast to these later findings, in humans, an inhibition of sperm motility in normal semen has been observed following MT administration *in vitro*.

3.5.4 Melatonin and pregnancy

3.5.4.1 Maternal serum melatonin concentrations, placental passage of melatonin and melatonin functions in the fetus

In humans, the night-time serum concentrations of MT increase already after 24 weeks of gestation, but exhibit significantly higher levels after 32 weeks compared to non-pregnant women. On the 2nd day of puerperium, these values drop again to non-pregnant levels [345, 346]. In rats, a similar increase of maternal night-time MT levels toward the end of pregnancy was observed. The circulating rat maternal MT is most likely of maternal pineal gland origin increased via the action of yet unidentified placental hormones [295]. In different species, including humans, it has been demonstrated that MT crosses the placenta, and that fetal circulating MT is mainly of maternal origin (see Figure 3.8; [347, 348, 349]). Interestingly, MT concentrations in human umbilical arteries are generally higher than those in the corresponding veins at normal vaginal delivery, indicating that the fetus may be capable of producing MT [345]. Indeed, rat fetal brain was shown to synthesize MT. Nevertheless, fetal plasma MT is very low after maternal pinealectomy [350]. After birth, the full-term neonate does not produce MT for 2-4 month, resulting in a transient absence of MT [351].

Several studies suggest that via maternal MT photoperiodic information is provided to the fetus thereby synchronizing maternal and fetal physiology (see Figure 3.8; [352, 353, 354, 355, 356, 357, 358]). Maternal MT may also modulate fetal clock gene function via MT1 in the fetal SCN [359], could be one of the factors that regulate fetal rapid eye movement and non fetal rapid eye movement cycles [360], and may influence fetal gonadal growth [361, 362].

Importantly, authors have failed to find adverse effects of MT on prenatal growth, viability or morphology of the conceptus, even when exceptionally high doses of MT (up to 200 mg / kg / day) were used to treat pregnant rat dams from gestational day 6 –19 [363].

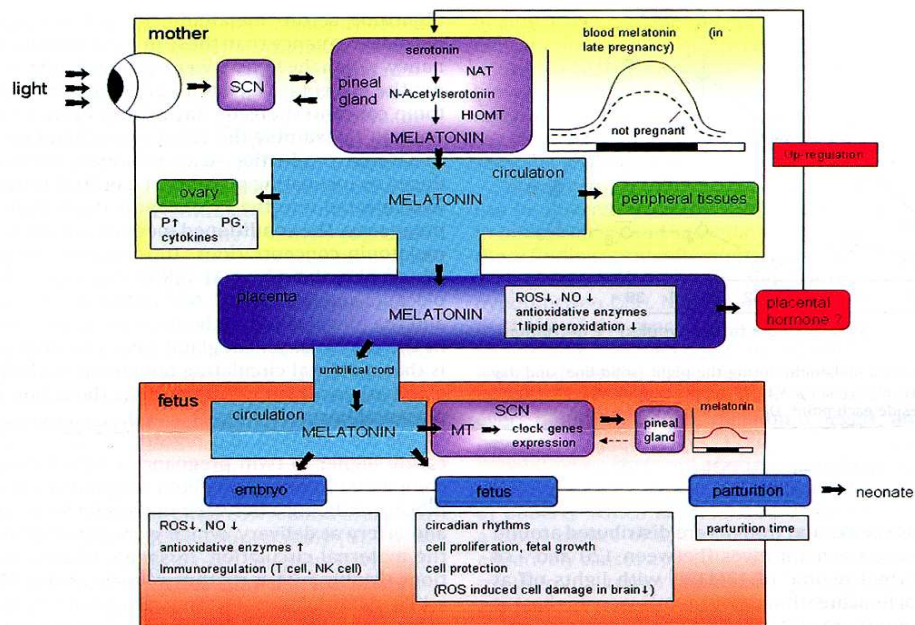


Figure 3.8 Schematic depictions of the proposed pathway and role of MT in the maternal-placental-fetal system. Photoperiodic information detected by the mother's eye is transduced via SCN to the pineal gland to regulate the rhythmic melatonin secretion. Pineal synthesis of melatonin from serotonin by enzymes (NAT, HIOMT) is up-regulated during pregnancy, probably by a hormone of placental origin. Melatonin is released into the maternal circulation and transported to the peripheral tissues including the placenta. Melatonin crosses the placenta without modification and enters the fetal circulation, where it can act in a variety of ways. SCN: suprachiasmatic nucleus; NAT: N-acetyltransferase; HIOMT: hydroxyindole-O-methyltransferase; P: progesterone; PG: prostaglandine; PRL: prolactin; ROS: reactive oxygen species; NO: nitric oxide; MT: melatonin receptor (from: [295]).

3.5.4.2 Melatonin receptors in placenta and uterus

The mRNA of MT1 and MT2 was found in first trimester human placenta [364]. Furthermore, MT1 and MT2 and ROR α 1 MT receptors were demonstrated at mRNA and protein level in isolated and in vitro differentiated villous trophoblast from term human placenta as well as in JEG-3 and BeWo placental choriocarcinoma cells. Immunohistochemical analysis of term placentas suggested localization of MT1 and MT2 in cytotrophoblast and syncytiotrophoblast (STB) as well as in endothelial cells surrounding the fetal capillaries and in the villous mesenchymal core, while ROR α 1 was not expressed in endothelial cells [44]. An MT agonist (6-chloromelatonin) inhibited, in a dose-dependent manner, forskolin-stimulated human chorionic gonadotrophin (hCG- β) secretion in JEG-3 and BeWo cells. This effect of 6-chloromelatonin was abolished by pertussis toxin (PTX), suggesting that MT regulates hCG- β production by an action involving an inhibitory Gi/o protein [365]. This expression of MT receptors in the human, but also rat placenta and the ability of MT to stimulate human chorionic gonadotropin and to downregulate rat placental

lactogen II mRNA levels, suggest that MT not only crosses the placenta, but plays a functional role in feto-placental development [45, 364, 44].

MT1 and MT2 receptors are also expressed in human [366] and rat myometrial cells [367]. Recent studies suggest a function of MT in parturition. MT2 expression was found markedly elevated in samples from pregnant women who had entered labor, as compared to matched nonlaboring pregnant women. In *in vitro* experiments, MT synergized with the action of oxytocin to promote muscle contractions and gap junction activity that is important in the coordination of myometrial contractions [305]. Such a synergy *in vivo* would promote coordinated and forceful contractions of the late term pregnant uterus necessary for parturition.

3.5.4.3 Pregnancy, oxidative stress and melatonin

Pregnancy is accompanied by a high metabolic demand and elevated requirements for tissue oxygen. This increased oxygen demand augments the rate of production of ROS. Especially the placenta is a major source of oxidative stress during pregnancy. Elevated generation of ROS by placental mitochondria during the first trimester has been demonstrated [368]. As the placenta is rich in polyunsaturated fatty acids, it is also an abundant source of lipid peroxides, which are secreted into the maternal circulation. However, in normal pregnancy, placental lipid peroxide production is controlled by placental anti-oxidant enzymes [369]. Major anti-oxidant enzymes such as superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, glutathione S-transferase and glucose-6-phosphate dehydrogenase are all present in the placenta and in normal pregnancy, placental anti-oxidant defenses are considered sufficient to control lipid peroxidation.

As outline (see 3.1.1), MT works in several ways to reduce oxidative stress. Elevated levels of maternal serum MT in pregnant women may therefore have an essential role in reducing placental oxidative stress. Additionally, there is evidence for production of MT in human placenta; NAT and HIOMT, important MT synthesizing enzymes were found expressed and active in total term placental tissue [44]. Expression of the enzymes in isolated and *in vitro* differentiated villous trophoblast as well as in JEG-3 and BeWo placental choriocarcinoma cells, both *in vitro* models of human trophoblast, indicated that villous trophoblasts could participate in the production of the enzymes. Moreover, mRNA of the 2 enzymes was also demonstrated in the first trimester human placenta [364]. This locally produced MT might aid to the anti-oxidative protection of the placenta. Although this is mainly a hypothesis, several publications argue for this function:

Spontaneous abortion is estimated to occur in 15 – 20 % of identified pregnancies and several studies have implicated systemic and placental oxidative stress in the

pathophysiology of abortion [297, 370, 371]. In pregnant rats, pinealectomy lowered circulating MT levels and led to abortion [372]. Circulating and placental MT might add to antioxidative protection thereby preventing abortion. Furthermore, MT has immunomodulatory functions (see 3.1.2), could influence cells of the maternal immune system and thereby support the tolerance mechanisms that allow survival of the fetus in the maternal uterus [295]. In addition, MT may contribute to the maintenance of early pregnancy by stimulating progesterone production in the ovary, most likely in luteal cells [303, 295].

Maternal undernutrition produced a fall in rat placental efficiency, disproportionate intrauterine growth retardation and a reduction in birth weight [373]. However, maternal treatment with MT improved placental efficiency, restored birth weight, and it increased the expression of placental manganese superoxide dismutase (Mn-SOD) and catalase, two anti-oxidant systems. Importantly, blood flow in most circulations can be regulated by the balance between free radical production and the bioavailability of nitric oxide (NO). Reductions in blood flow may therefore occur as the result of an increase in O₂ production and/or a fall in anti-oxidant capacity, while an increase in anti-oxidant capacity and/or a fall in free radical production will result in vasodilatation. Especially during pregnancy, NO is indispensable for the maintenance of blood flow in the placental and umbilical circulations providing appropriate nutrient and oxygen delivery to the fetus [374]. The observed protective function of MT in undernourished pregnancy may consequently be related to an increase in the endogenous placental anti-oxidant capacity, shifting the vascular oxidant ratio toward dilatation and, thereby, improving placental efficiency and maintaining perfusion and nutrient and oxygen delivery to the fetus. Alternatively, MT might influence the density of nutrient transporters at the materno-fetal interface or mediate changes in concentration gradients. It is known that the human and rat placenta express receptors for MT [45, 364, 44], but whether MT has any receptor-mediated or indirect effects on placental transporters or in the concentration gradient of glucose either in normal or complicated pregnancy remains unknown.

PE is a condition of elevated oxidative stress [375]. A significant rise in lipid peroxidation levels in the placenta of PE-affected women has been shown [376], while significant lower levels of anti-oxidant systems are found in the circulation and the placentas of PE-affected women [377, 378, 379, 380]. The rise in placental and systemic oxidative stress may be involved in the pathogenesis of PE (see 3.4.2.4). Women with severe PE were found to have lower night-time serum MT levels than those with mild or no PE at 33 weeks of pregnancy [345] and a recent report noted that anti-oxidants (vitamin C and E) effectively prevented PE in women at increased risk of disease [381]. It is, however, currently completely speculative at which site MT, which exhibits a variety of interesting characteristics (e.g. anti-oxidant, cardiovascular regulator, anticonvulsant) could interact with the mechanisms of disease

functioning in PE [295, 296]. However, MT may protect the placenta from free radical-induced injury by increasing the activity of anti-oxidant enzymes. This concept was tested in pregnant women before they underwent voluntary disruption of pregnancy between 7 and 9 wk of gestation. Indeed, the oral MT administration (6 mg) was reflected by markedly increased MT concentrations in maternal serum and in chorion and an induction of GSH-Px activity in chorionic homogenates [149].

In summary, MT synthesising enzymes and MT receptors are present in the human placenta, and MT from the maternal circulation reaches the placenta and is transferred to the fetus. From the current literature it can be concluded that MT might mediate important functions in the placenta, either directly as an anti-oxidant or indirectly via its receptors. However, much research is required to investigate these functions under physiologic and pathologic (e.g. PE) conditions.

4 AIM

During pregnancy, circulating maternal MT enters the placenta and is transferred to the fetus thereby providing important photoperiodic information to the offspring [295].

In addition, an interaction between the placenta and MT must be assumed. Placental cells facing the maternal circulation (i.e. syncytiotrophoblasts) express the G-protein coupled MT-receptors MT1 and MT2 as well as a nuclear receptor of the RZR/ROR family [364, 44]. MT, known to influence the production and secretion of many hormones, has been demonstrated to inhibit the expression of rat placental lactogen [45] and to modulate the secretion of human chorionic gonadotropin hCG [364, 365]. Via multiple pathways, MT functions as an effective anti-oxidant and in human placental chorion, stimulation of the anti-oxidant enzyme GSH-Px by MT has been shown [149]. MT is an oncostatic agent and has exhibited anti-mitogenic MT2 receptor-mediated effects in placental choriocarcinoma cell lines (JEG-3 and JAR [382, 383]. Moreover, MT possesses anti-inflammatory, cyto-protective, vasomotor and immunological properties that have so far not even been investigated in human placenta. These results suggest that MT via receptor-mediated actions plays an important role in the regulation of trophoblast development (e.g. proliferation, differentiation, and apoptosis) and endocrine function (e.g. hormone production). However, the receptor-mediated effects of MT in placental chorionic cells have not been investigated systematically.

In addition, pregnancy-related pathologies, such as PE or abortion are associated with excessive oxidative stress that is no longer counterbalanced by endogenous anti-oxidative systems. Treatment of PE with pharmacological doses of MT as a potent (direct) anti-oxidant has been suggested [311] and at least in women undergoing an *in vitro* fertilization and embryo transfer, pharmacological concentrations of MT were found to double the fertilization

and pregnancy rate [295]. Although even a pharmacological dose of MT given during pregnancy did not influence growth, viability and morphology of rat offsprings [363], a profound knowledge of the effects of MT mediated via placental receptors is of advice before applying MT during pregnancy.

Consequently, it is of major importance to establish *in vitro* systems (e.g. choriocarcinoma cell lines), where expression and function of placental MT receptors in human chorionic trophoblast cells can be studied. Investigations of MT receptors at the protein level are, however, often limited by a lack or reduced number of commercially available anti-MT1 antibodies.

At the beginning of this study, anti-MT1 antibodies available for disposal were Ab-13035 and Ab-13036 from Abcam and sc-13179 from Santa Cruz. Furthermore, the choriocarcinoma cell line BeWo has been demonstrated to express MT1 mRNA as well as protein [365, 44].

The aim of this work was to:

- 1. Characterize and compare expression and localisation of MT1 protein by western blotting and immunofluorescence studies in the human choriocarcinoma BeWo cell line using several commercially available anti-MT1 antibodies (Ab-13035, Ab-13036, sc-13179).**
- 2. Generate stably transfected BeWo cells overexpressing MT1 receptor that allow for analysis of anti-MT1 antibody-specificity.**

5 METHODS AND MATERIALS

5.1 Tissue and cell culture

5.1.1 General remarks

All solutions and materials in direct contact with cells are sterile (autoclaved, heat-sterilized, sterile-filtered or sterilised with gamma radiation). 70 % EtOH is used to wipe surfaces in contact with the tissue plates. The handling of cells occurs in a laminar flow. Unless stated otherwise, all media and solutions used in cell culture should be pre-warmed to 37 °C. Cells are grown in an incubator with 5 % (v/v) CO₂ in the atmosphere. In addition, humidity and temperature within the incubator are adjusted to 95 % (v/v) and 37 °C respectively to ensure optimal growth of cell.

pH (power of the hydrogen) measures of the acidity/alkalinity of a solution. The pH scale is an inverse logarithmic representation of hydronium ion [H₃O⁺]. Most substances have a pH in the range 0 – 14; when the pH level is 7.0, it is defined as neutral [H₃O⁺ = OH⁻]. Extracellular pH (of media and buffers) affects many intracellular properties, including pH, cell metabolism, glucose transport, and the ATP/ADP ratio [384, 385]. The optimum pH for cell growth has traditionally been determined by cell growth experiments, with most cell lines growing well at approximately pH 7.4 (range 7.2 – 7.4). Therefore, cell culture media are usually buffered to pH 7.4 by a carbonate buffer system (components of the buffer system: CO₂ as a weak acid, HCO₃⁻ as a correspondent salt), with HCO₃⁻ provided by the medium. To complete the buffer system, cells are kept in an incubator with 5 % (v/v) CO₂ in the atmosphere. To estimate pH and recognize deviations of pH caused by cell metabolism (or exogenous influences, such as alterations in pCO₂), cell culture media and other sterile solutions used in cell culture often contain the pH indicator phenol red. Phenol red is red at pH 7.4, turns orange below pH 7.0, yellow below pH 6.5 and purple above pH 7.8. Cell growth and metabolic activity, but also increased levels of pCO₂ (> 5 %) can result in acidification of the medium, while reduction of pCO₂ (< 5 %) results in an alkalinisation of the medium. Therefore, cell culture media have to be changed regularly (every 2 – 3 day) and pCO₂ of the incubator needs to be controlled. To ensure correct pH values of buffers prepared in the laboratory (e.g. phosphate buffered saline, PBS), the pH was measured with a pH meter according to the instructions of the manufacturer.

Osmolality (unit: Osm/kg) is a measure of the osmoles of solute per kilogram of solvent. **Osmole (Osm)** is a non-SI unit of measurement that defines the number of moles of a chemical compound that contribute to a solution's osmotic pressure. Human plasma has an osmolality of about 290 mOsm / kg (range: 280 – 303 mOsm / kg); that is also assumed to be the optimum for most cells *in vitro*. Because hypo-osmolality (below this range) or hyper-osmolality (above this range) causes serious damage to the cells, osmolality of solutions

prepared in the laboratory was routinely measured using an osmometer, which uses the principle of freezing-point depression.

5.1.2 Materials

- Laminar Flow (Herasafe KSP 12, ThermoScientific)
- Incubator (Cytoperm2 / Heraeus) set to 5 % (v/v) CO₂, 95 % (v/v) humidity and 37 °C temperature
- Centrifuge (Rotixa/RP, Hettich) used at 200 x g (i.e. 1000 rpm)
- Inverted Microscope (Olympus, CK2), equipped with 10x, 20x and 40x objectives (total magnification: 100x – 400x)
- Waterbath (GFL 1083) set to 37 °C, A.bid. supplied with Aqua Resist (VWR Int. 462 – 7000)
- Osmometers (Osmomat 030 - D, Gonotec)
- Kryostorage system (-196 °C, K8, Kendro)
- 25 cm² canted neck tissue culture flasks, sterile (IWAKI 3103 – 025)
- 75 cm² canted neck tissue culture flasks, sterile (IWAKI 3110 – 075)
- 50 ml centrifuge tubes, sterile (TPP 91051)
- 1.5 ml cryogenic vials, sterile (Nalge Company 5000 – 1020)
- 10 ml single - use syringes (B. Braun Melsungen AG H4606108V)
- Disposable sterile syringe filters, 25 mm, 0.20 Micron, cellulose acetate membrane acrylic (IWAKI 2052 – 025)
- 10 + 25 ml glass pipettes, sterile
- 230 mm glass pasteur pipettes, sterile (VWR 612 – 1702)
- Gloves (e.g. Latex-gloves Peha-soft; Hartmann 942161)
- Scissors and forceps
- Ice bucket

5.1.3 Reagents

- Dulbecco's modified eagle medium = DMEM - high glucose, sterile - filtered (Sigma D - 5796), stored at 4 °C
- Advanced DMEM/F - 12 (Gibco/Invitrogen 12634 – 010), stored at 4 °C
- Foetal bovine serum = FCS, sterile (Gibco/Invitrogen 10270 – 106), 500 ml treated at 56 °C for 30 min, (heat inactivation is performed to destroy complement, and to ensure that the cells will not be lysed by antibody binding), stored in ready - to - use aliquots (50 ml) at -20 °C
- 200 mM Glutamine (GlutaMAX-I Gibco/Invitrogen 35050 – 038), sterile, stored in ready - to - use aliquots (5.5 ml) at -20 °C

- Liquid Antibiotic Mixture (PSN, Gibco/Invitrogen 15640 – 055), 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 °C
- Dimethyl sulphoxide = DMSO, sterile (Sigma D2650), stored at rt
- 10 x Trypsin/EDTA solution, sterile (Gibco/Invitrogen 15400 – 054), containing 5.0 g porcine trypsin and 2g ethylenediaminetetraacetic acid EDTA .4Na per liter of 0.9 % sodium chloride, stored at -20 °C
- Phenol red sodium salt (Sigma P4758 ; Phenolsulfonephthalein sodium salt), stored at rt
- KCl (Merck 4936.1000)
- NaCl (Merck 1.06404.1000)
- KH_2PO_4 (Merck 1.04873.1000)
- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (1.06580.1000)
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck 1.02382.1000)
- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Merck 1.05886.1000)

5.1.4 Solution and media

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

- Dissolve the following salts in 800 ml A.bid.
 - o 0.20 g KCl, final concentration 2.7 mM
 - o 8.01 g NaCl, final concentration 137 mM
 - o 0.21 g KH_2PO_4 , final concentration 1.5 mM
 - o 1.44 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 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 osmolality (~290 m osm / kg)
- Sterilize by autoclaving
- Store at rt

1M CaCl_2 solution

- Dissolve 14.698 g CaCl_2 in a final volume of 100 ml A.bid.
- Sterilize by autoclaving
- Store at rt

0,5M MgCl_2 solution

- Dissolve 10.19 g MgCl_2 in a final volume of 100 ml A.bid
- Sterilize by autoclaving

- Store at rt

PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (PBS⁺⁺, sterile)

- To 1 l PBS, sterile, add
- 1 ml 1 M CaCl₂ solution final concentration 1 mM, sterile
- 1 ml 0.5 M MgCl₂ solution final concentration 0.5 mM, sterile
- Store at 4 °C

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

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

Trypsin/EDTA working solution (1x), sterile

- 100 ml 10x 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
- Aliquot in 50 ml tubes and store working aliquot at 4 °C
- For long term storage keep aliquots at - 20 °C

Culture medium for BeWo cells

- 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 antibiotic mixture, final concentration 50 µg / ml penicillin, 100 µg / ml streptomycin, 50 µg / ml neomycin, respectively
- Store at 4 °C

Freezing medium for BeWo cells

- 40 ml culture medium
- 5 ml FCS, final concentration 19 % (v/v)
- 5 ml DMSO, final concentration, 10 % (v/v), used as a cryoprotective agent
- Store at 4 °C

Transport medium for placenta

- 500 ml advanced DMEM/F12, sterile
- 5 ml FCS, final concentration 1 % (v/v)
- 5 ml Glutamine, final concentration 2 mM
- 0.5 ml liquid antibiotic mixture, final concentration 50 µg / ml penicillin, 100 µg / ml streptomycin, 50 µg / ml neomycin)
- Store at 4 °C

5.1.5 Collection and preparation of placental tissue

Human term placentas were obtained following elective caesarean section of uncomplicated pregnancies at 38 – 40 weeks gestation from the Clinic for Gynaecology and Obstetrics, Medical University of Vienna. The women had no pre-existing medical conditions.

Within 15 min after delivery, chorionic tissue of 3 – 4 areas of each placenta (~1 cm³) was randomly sampled. Villous samples were taken midway between the chorionic and basal plates from areas free of visible infarction, calcification or hematoma, using scissors and forceps. After a brief rinse in ice-cold PBS⁺⁺, samples were placed into ice-cold transport medium and rapidly transferred to the lab (< 15 min). Tissue (~ 0.2 – 0.5 cm³) was either immediately frozen in liquid nitrogen or stored at -80 °C for RNA or protein preparation (section 5.7 and 5.2.5 respectively). When used for immunofluorescence microscopy, tissue was further processed as described in (section 5.5.4).

5.1.6 Cell lines**BeWo cells (b24 clone)**

The BeWo cell line has been derived from a human choriocarcinoma [386]. The subclone b24 was obtained from the parental line by limited dilution and displays many morphological and biochemical properties common to placental trophoblasts [387, 388]. BeWo cell clone b24 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).

HOS, MG63, OVCAR and TC71 cells

The Human osteosarcoma cell line HOS and MG63 as well as the ovarian carcinoma OVCAR-3 and the Ewing's sarcoma cell line TC71, that have been demonstrated to express melatonin receptor MT1 [389, 390] were kindly provided by A.o.Prof. Dr. Theresia Thalhammer.

5.1.7 Cell culture methods

Feeding

- Examine cells (cultured in flasks) using an inverted microscope (magnification 100 – 200x) to estimate cell growth or discover potential infections
- Transfer healthy cells to laminar flow (discard flasks containing cells infected by fungi or bacteria)
- Aspirate medium and wash cells once with PBS to remove cell debris and dead cells (5 ml / 25 cm² flask and 15 ml / 75 cm² flask)
- Add fresh medium to the cells (5 ml / 25 cm² flask and 15 ml / 75 cm² flask)
- Return cells to incubator

Passaging of cells (Cell - splitting)

- Transfer healthy cells to laminar air flow
- Aspirate medium and wash cells twice with PBS to remove cell debris, dead cells and trypsin inhibitor contained in the medium.
- Add trypsin - EDTA working solution (0.5 ml / 25 cm² flask 1.0 ml / 75 cm² flask)
- Place in incubator for ~5 min at 37 °C (depending on the cell line and degree of confluency, the incubation time has to be expended up to 15 min).
- Examine cells by light microscopy; cells should round up and detach from the substrate.
- When cells are detached, add culture medium (5 ml / 25 cm² and 75 cm² flasks) and re-suspend cells by pipetting up and down twice.
- Transfer aliquots of the cell suspension to new flask containing 5 ml / 25 cm² or 10 ml / 75 cm² culture medium (maximum splitting - rate for BeWo cells is 1:5)
- Return cells to incubator.

Freezing of cells

- Culture cells in 75 cm² flask to 80 % confluency
- Transfer healthy cells to laminar flow
- Aspirate medium and wash cells twice with PBS to remove trypsin inhibitor contained in the medium
- Add trypsin /EDTA working solution (1.0 ml / 75 cm² flask)
- Incubate for ~5 min (or longer)
- Examine cells by light microscopy; cells should round up and detach from the substrate
- Upon detachment, add culture medium (10 ml / 75 cm² flask) and resuspend cells by pipetting up and down twice

- Transfer cell suspension to 15 ml centrifuge tube
- Pellet cells by centrifugation at 200 x g for 5 min at 4 °C
- Discard sup, add 5 ml freezing medium (pre-cooled to 4 °C), suspend cells by pipetting up and down twice
- Transfer cell suspension to cryotubes (1 ml / tube on ice)
- Keep tubes on ice, and transfer to -80 °C
- Transfer to -196 °C (liquid nitrogen storage container) the next day

5.2 Preparation of cell and tissue homogenates

5.2.1 Materials

- Microcentrifuge (Eppendorf, 5415D), cooled to 4 °C
- Ultra Turrax (Janke & Kunkel)
- Scintillation tubes (20 ml), remove top-part (narrow neck) of the tube with e.g. a knife
- Cell scraper (Iwaki 9010 – 230)
- Reaction tubes, 1.6 ml (Biozyme 710160)
- Mortar and pestle, cooled
- Ice bucket with ice
- Liquid nitrogen (wear protective glasses and gloves)

5.2.2 Reagents

- TX-100 (Merck 11869 – 1000)
- Protease inhibitor cocktail (Sigma - P8340) stored in 50 µl aliquots at -20 °C, used in a final dilution of 1:100 and containing:
 - o AEBSF - [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride], inhibits serine proteases
 - o Aprotinin, inhibits serine proteases
 - o Bestatin hydrochloride, inhibits aminopeptidases
 - o E-64-[N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide], inhibits cysteine proteases
 - o Leupeptin hemisulfate salt, inhibits serine and cysteine proteases
 - o Pepstatin A, inhibits acid proteases

5.2.3 Solutions

PBS (1x), sterile

- See 5.1.4

Lysis buffer

- To 200 ml PBS add 0.2 ml TX-100 / final concentration 0.1 % (v/v)
- Store at rt

5.2.4 Preparation of cell lysates for western blotting

- Grow cells to 80 – 90 % confluence
- Transfer flasks with cells from the cell culture lab to the bench (on ice)
- Wash cells with 5 ml / 15 ml PBS (4 °C) per 25 cm² / 75 cm² flask, respectively (2x), aspirate buffer
- Add 0.5 / 1 ml PBS ml per 25 cm² / 75 cm² flask, respectively
- Harvest cells with a cell scraper and transfer cell suspension into microfuge tubes (on ice)
- Collect cells by centrifugation (200 x g, 5 min, 4 °C), discard sup
- Add lysis buffer (ice-cold) containing protease-inhibitor cocktail (see 5.2.2) to pellet: 0.2 ml / 0.6 ml per 25 cm² / 75 cm² flask, respectively
- Vortex; rotate at 4 °C for 30 min
- Remove insoluble material by centrifugation in microcentrifuge (12.000 x g, 5 min, at 4 °C)
- Transfer supernatant into fresh tube, discard pellet
- Aliquote lysates (100 µl) and store lysates at -20 °C (for long time storage at -80 °C), avoid repetitive thawing and freezing (degradation of proteins!)

5.2.5 Preparation of lysates from placental tissue for western blotting

- Placental chorionic tissue (quickly frozen in liquid nitrogen and stored at -80 °C, see 5.1.5)
- Use 1 – 2 g of tissue (keep frozen)
- Grind material using mortar and pestle in the presence of liquid nitrogen
- Transfer material into a wide-neck scintillation tube and allow nitrogen to evaporate (but do not allow tissue to thaw)
- Add 2ml / 1g placenta of lysis buffer containing protease-inhibitor cocktail (see 5.2.2)
- Homogenate with Ultra Turrax (for 1 min, raise one level every 10 sec; from lowest to highest level)
- Incubate at 4 °C (30 min)

- Transfer to microfuge tube (1 ml / tube)
- Centrifuge in microcentrifuge (12.000 x g, 5 min, at 4 °C)
- Transfer sup into new tube
- Aliquote lysates (100 µl) and store lysates at -20 °C (for long time storage at -80 °C), avoid repetitive thawing and freezing (degradation of proteins!)

5.3 Determination of protein concentration in cell lysates with the Bradford assay

5.3.1 General remarks

The Bradford assay [391] is very fast and very accurate. It is especially recommended when assessing protein concentrations for gel electrophoresis. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G - 250 shifts from 465 nm to 595 nm when binding to proteins occurs. As the absorption a spectrum of the two forms of the dye overlap, the assay performs linearly only over short concentration stretches. Since the assay responds non-linearly, it is highly important to lock down the zero point. At least two buffer blanks should be performed.

The choice of standard is crucial to the success of the assay. It has been noted that bovine serum albumin (BSA) has a double than “normal” response in the assay and may not always be suitable. Therefore Immunoglobulin G (IgG) is the preferred standard for the assay.

The dye used in the assay binds to quartz cuvettes quite strongly. Therefore, glass or plastic cuvettes should be utilized.

5.3.2 Materials

- Foaldded filters (Machery - Nagel 640 m)
- Reaction tubes, 1.6 ml (Biozyme 710160)
- Spectrophotometer (Hitachi U - 2800A spectrophotometer)

5.3.3 Reagents

- Coomassie brilliant Blue G - 250 (LKB 1840 – 102)
- Ethanol (Merck 1.00983 .1000)
- Ortho-Phosphoric acid, 85 % (Merck 573.1000)
- Human IgG (Endobulin S/D, Baxter)

5.3.4 Buffers

Bradford reagent

- Dissolve 100 mg Coomassie Blue G - 250 in 50 ml ethanol (95 % (v/v)) by stirring
- Add 100 ml (85 % (w/v)) ortho-phosphoric acid, stir on at rt

- Dilute with A. bid. to 1 liter when the dye has completely dissolved, stir
- Filter through Whatman #1 paper
- Store at rt, protect from light (colour should be light brown; if it turns blue, discard)

hIgG standard solution

- A stock solution of hIgG in A.bid (100 mg / ml is stored at -80° in 100 µl aliquots in A.bid. to a concentration of 1 mg / ml
- Aliquot (100 µl) and store at -20 °C

5.3.5 Assay

- Pipette hIgG standard solution (0, 5, 10, 20 µl) in duplicate into tubes
- Pipette aliquots of samples of interest in duplicate into tubes (sample volumes need to be determined). Empirically, try 5 – 20 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 at 595 nm in a spectrometer (turned on 15 min before use)
- Calculate protein concentration in samples using the standard curve

5.4 Sodium dodecylsulfate polyacrylamide Gel Electrophoresis (SDS PAGE) and Western blotting

5.4.1 General remarks

SDS-PAGE [392] is a technique used to separate proteins according to their electrophoretic mobility. Electrophoretic mobility is a function of molecular weight, higher order protein folding, posttranslational modification, protein charge, etc.

The addition of the anionic detergent SDS, to the protein samples results in denaturation of secondary and non-disulfide-linked tertiary structures and applies a negative charge to each protein in proportion to its mass. Consequently, proteins are linearized and separated strictly by molecular pore size. Polyacrylamide (PA) gels are synthetic gels that are thermostable, transparent, strong, relatively chemically inert and can be prepared with a wide range of average PA gel (3 – 30 %) prepared with a pH 8.8 Tris buffer. Discontinuous electrophoresis combines two gels of different pore sizes. The stacking gel is a large pore PA gel (4 %), prepared with a Tris buffer (pH 6.8). This condition allows protein concentration and a thin starting zone [420]. The subsequent separating gel is a PA gel of variable Gel smaller pore size. The presence of a reducing agent in the sample buffer (e.g. β-mercaptoethanol) and boiling additionally ensures

disulfide bond destruction and destruction of tertiary and quaternary structure (reducing SDS-PAGE).

Western blotting (Immunoblotting) [393] is a method to detect a specific protein in a sample of tissue or cell homogenate. Following gel electrophoresis (to separate denatured proteins by molecular size), proteins are transferred to a membrane like polyvinylidene difluoride (PVDF) or nitrocellulose, where they are probed using antibodies specific to the target proteins. One method for transferring proteins from gel to membrane is electroblotting using electrocurrent to pull proteins from the gel into the PVDF/nitrocellulose membrane. As the result of the blotting process, the proteins are finally exposed on the surface of the membrane maintaining the organization they had within the gel. PVDF and nitrocellulose membrane both bind protein non-specifically by hydrophobic as well as charged interactions. Nitrocellulose is more fragile, but cheaper than PVDF membranes. The uniformity and effectiveness of transfer of proteins from the gel to the membrane can be checked by staining all proteins transferred to the membrane non-specifically with Ponceau S and those remaining in the gel with Coomassie blue dyes.

Coomassie Blue staining of gels [394] is based on the binding of the dye Coomassie Brilliant Blue R250, which binds non-specifically to virtually all proteins. (Sensitivity: 10 µg of protein). It is very convenient and can be used to check for transfer-efficiency after blotting of proteins from gel to membranes. The gel is soaked in a solution of the dye and any dye that is not bound to protein diffuses out of the gel during the destain steps. The proteins are detected as blue bands on a clear background.

Ponceau S [395] is a sodium salt of a diazo-dye that is used for a rapid and reversible detection of protein bands on PVDF and nitrocellulose membranes. The stain is easily reversed with PBS washes, facilitating subsequent immunological detection.

Enhanced chemoluminescence (ELC): immobilized specific antigens recognized by HRP- labelled secondary antibodies are detected by enzymatic generation of light from a substrate. The emitted light can be detected on autoradiography films.

5.4.2 Materials

- Gel electrophoresis apparatus (LKB 2050 Midget)
- Power supply for electrophoresis apparatus (LKB 2297 Macrodrive 5)
- Blotting apparatus (PeqLab, PerfectBlue Semi-Dry Electroblotter)
- Power supply for blotting apparatus (PeqLab Consort EV231)
- Scanner (Hp scanjet 5470c)
- Film-cassette (DuPont)
- Film-Developing machine Agfa GP 1000

- Heat-block, adjusted to 95 °C

5.4.3 Reagents

- Acrylamide Bis solution 40 %, 37.5:1 (Bio - Rad 161 – 0148) for separation gel
- Acrylamide Bis solution 40 %, 19:1 (Bio - Rad 161 – 0144) for stacking gel
- Sodium dodecyl sulfate (SDS; Sigma L - 3771)
- Ammonium persulfate (AMPS; Sigma A - 3678)
- Tetramethylethylenediamine (TEMED; Bio-Rad 161 – 0801)
- 2-Mercaptoethanol (Sigma M - 7154)
- Glycerol ;about 87 % (Merck 1.04094.0500)
- Bromophenol blue (LKB 1840 – 901)
- Tris(hydroxymethyl)aminomethane (Merck 1.08382.1000)
- 1-Butanol (Merck 1990)
- Hydrochloric acid, fuming 37 % (Merck 1.00314.1000)
- Coomassie brilliant blue G250 (LKB 1840 – 102)
- Protein molecular weight markers:
 - o Precision Plus Protein Kaleidoscope Standards (Bio-Rad 161 – 0324), 6 µl / Minigel (6,5 – 204 KD)
 - o Page Ruler Prestained Protein Ladder (Fermentas SM 0671), 5 µl / Minigel (10 – 170 KD)
 - o Spectra Multi color Broad Page Protein Ladder (Fermentas SM 1841), 10 µl / well (10 – 260 KD)
- Methanol (Fisher Scientific M/4000/17)
- Glycine (Merck 1.04169.1000)
- Acetic acid (Merck 8.18755.2500)
- Dry milk powder (Fixmilch Instant, Maresi)
- Ponceau S, practical grade (Sigma P - 3504)
- Tween 20 for electrophoresis (Sigma P - 5927)
- PVDF transfer membranes, 0.45 µm (Pall Corp. Pall BioTrace™ P/N 66543)
- Extra Thick Blot Paper, Miniblot size 7 x 8.4 cm (Bio – Rad 170 – 3966)
- Amersham ECL Plus Western Blotting Detection Reagents (PIERCE 34080) or supersignal west pico chemiluminescent substrate (Thermo Scientific 34080)
- Amersham Hyperfilm ECL (GE Healthcare 28 – 9068 – 36)
- Saran wrap
- G153 Developer and G353/G354 Fixer (Agfa)
- Primary and secondary antibodies (see Table 5.1)

Table 5.1 Primary and secondary antibodies (ABs) used to detect MT-1 by western blotting

	Primary antibody	Secondary antibody
Antibody set I	Anti-Melatonin receptor 1A, affinity purified polyclonal AB, raised in rabbit against a peptide that represents an epitope in the 2nd cytoplasmic loop of MT1 Abcam, ab13035 Dilution 1:500 (2 µg / µl)	Anti-rabbit IgG (whole molecule), affinity isolated, peroxidase conjugated AB, produced in goat Sigma, A – 0545 Dilution 1:20.000
Antibody set II	Anti-Melatonin receptor 1A, affinity purified polyclonal AB, raised in rabbit against a peptide that represents an epitope in the 3rd cytoplasmic loop of MT Abcam, ab13036 Dilution 1:500 (2 µg / µl)	Anti-rabbit IgG (whole molecule), affinity isolated, peroxidase conjugated AB, produced in goat Sigma, A – 0545 Dilution 1:20.000
Antibody set III	Anti-Melatonin receptor 1A, affinity purified polyclonal AB, raised in goat against an N-terminal peptide of MT-1 Santa Cruz, sc - 13179 Dilution 1:200 (1 µg / µl)	Anti-goat IgG-HRP, affinity purified peroxidase conjugated AB, produced in donkey Santa Cruz, sc - 2020 Dilution 1:5.000
Blocking peptide	sc-13179P	
Antibody set IV	Anti-HA tag monoclonal AB, produced in mouse Gene Tex, GTX21424 Dilution 1:1.000 (1 µg / µl)	Anti-mouse IgG, affinity purified peroxidase conjugated AB produced in donkey Promega, W4021 Dilution 1:5.000

5.4.4 Solutions

10 % (w/v) AMPS solution

- Dissolve 1 g AMPS in 10 ml A.bid.
- Aliquote (1 ml) and store at -20 °C

10 % (w/v) SDS solution

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

M Tris-HCl, pH 8.8 (500 ml)

- Dissolve 181.7 g Tris in A.bid. (~450 ml)
- Adjust pH 8.8 with HCl
- Adjust to a final volume of 500 ml

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

- Dissolve 6.1 g Tris in A.bid. (~80 ml)
- Adjust pH 6.8 with HCl
- Adjust to a final volume of 100 ml

SDS-PAGE sample buffer (4x stock solution)

- 5 ml 0.5 M Tris-HCl, pH 6.8
- 3 ml Glycerol
- 2 ml β -mercaptoethanol
- 0.8 g SDS
- 5 mg Bromphenol blue
- Mix, dissolve by stirring, filter buffer and store in 1 ml aliquots at -20 °C

Sample buffer (1x)

- Mix 1 vol. 4x stock solution and 3 vol. A.bid.
- Final concentration
 - o Tris 62.5 mM
 - o Glycerol 6.5 % (v/v)
 - o SDS 2 % (w/v)
 - o β -mercaptoethanol 5 % (v/v)

Running buffer (10x stock solution)

- 30 g Tris (0.25 M)
- 144 g Glycine (1.92 M)
- 10 g SDS (1 % w/v)
- Dissolve and bring to a final volume of 1000 ml A.bid.
- Store at rt

Running buffer (1x)

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

Transfer buffer

- 5.8 g Tris (48 mM)
- 2.93 g Glycin (39 mM)
- 0.375 g SDS (0.0375 % (w/v))
- 200 ml MeOH (20 % (v/v))
- Dissolve and bring to a final volume of 1000 ml A.bid.
- Store at rt

Washing buffer

- PBS (1x (see 5.1.4) containing 0.1 % (v/v) Tween 20 (i.e. 1 ml Tween 20 + 999 ml PBS)

Ponceau red staining buffer

- 0.5 g ponceau red (0.1 % (w/v))
- 25 ml acetic acid (5 % (v/v))
- Dissolve and bring to a final volume of 500 ml A.bid.

Ponceau red destaining solution

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

Blocking buffer (=Blotto)

- 25 g dry milk powder (5 % (w/v))
- Dissolve and bring to a final volume of 500 ml PBS containing 0.1 % (v/v) Tween 20 (washing in buffer, see 5.4.4)

Coomassie blue staining solution

- 1.25 g Coomassie brilliant blue (0.25 % (w/v))
- Dissolve and bring to a final volume of 500 ml methanol:water:acetic acid (5:5:1)

Coomassie blue destaining solution

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

5.4.5 SDS-PAGE**Sample preparation**

- Equal amounts of protein were loaded from each sample calculated the determined protein concentration (10 – 40 µg / lane)
- To the sample volume (should be < 15 µl) add 5 µl of 4x SDS-PAGE sample buffer and if necessary - fill up to 20 µl with A.bid.
- Incubate samples for 3 min at 95 °C, spin briefly
- If not used immediately for SDS-PAGE, store samples at -20 °C
- Before use, heat again and collect fluid by centrifugation; proceed at (see 5.4.5)

Preparation of gels**(Preparation of separation gels)**

- 4 gels of desired percentage (see Table 5.2) poured at once.
- All materials used (plates, combs, spacers) must be clean (washed with detergent and water, followed by 70 % ethanol) and dry.
- Assemble aluminiumhydroxid plates, spacers (0.75 mm) and glass plates, respectively, to build the casting stand.
- Mix first 4 ingredients and add AMPS and TEMED immediately before pouring the gel, as they induce the gel polymerization
- Pour gel - solution into casting stand and leave place for stacking gel (~2 cm)
- Overlay each gel with n-Butanol (saturated with A.bid.) to ensure vertical surfaces
- Allow gels to polymerize for at least 1 h at rt

- Gels not used immediately were wrapped in saran wrap, placed in a wet-chamber and stored for a maximum 2 weeks at 4 °C

Table 5.2 Recipe for separation gels (volumes in ml, except for TEMED that is in µl)

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

(Preparation of stacking gel)

- Fix the number of gels required for the experiment in the gel electrophoresis apparatus
- Remove any remaining fluid on the surface of the separation gel using e.g. filter paper
- To prepare the stacking gel, mix required buffers and solutions as indicated in Table 5.3
- Overlay the separation gel with stacking gel solution and insert a cleaned comb (10 wells, 0.75 mm) carefully without including air bubbles
- Allow gel to polymerize for at least 30 min at rt
- Proceed with electrophoresis
- Remove combs

Table 5.3 Recipe for stacking gel

%	Acrylamide Bis solution (40 %) 19:1	Tris-HCl (0.5 M, 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

Electrophoresis

- Add 1x running buffer upper and lower to buffer reservoirs of electrophoresis apparatus
- Load samples and markers onto gels
- Fill empty sample wells with 1x SDS-PAGE sample buffer and run the gel at 150 V until the blue dye (Bromphenol blue) in the sample buffer has reached the bottom of the gel
- Turn off the power supply and disassemble the electrophoresis apparatus

5.4.6 Western blotting (Immunoblotting)**Transfer of proteins to PVDF/ membranes using a Semi-dry-Electro blotter**

- While running the electrophoresis, prepare 2 pieces of extra-thick per gel blot paper per gel with the same size as the gel.
- Submerge one PVDF membrane of same size first in MeOH (for 1 min), then in A.bid, and finally in blotting buffer (~10 min). As the PVDF membrane is extremely hydrophobic, it will not wet in aqueous solutions until pre-wetted with MeOH.
- After electrophoresis, remove stacking gel from separation gel and submerges gel in transfer blotter (see 5.4.4)
- Assemble blotting apparatus in the following way: Anode - filter paper - PVDF membrane - separation gel - filter paper - Cathode.
- The components of the stack should be wet, but there should not be excess fluid
- Avoid inclusion of air bubbles!
- Transfer proteins according to the following conditions:
 - o 90 min at 3 mA / cm²
 - o For 2 gels : 2 (gels) x 3 mA x 40 cm² / 240 mA (constant current)

Coomassie blue staining of gels

- Following transfer disassemble blotting apparatus Stain gel in Coomassie blue staining solution for at least 30 min at rt (shaking)
- Distain with Coomassie destaining solution for several hours at rt with several changes of buffer (shaking)
- Dry gel for at least 2 h at 80 °C under vacuum
- Scan gel to preserve the protein pattern; save as .tif-file

Ponceau staining of membranes

- After transfer, disassemble blotting apparatus (clean all components)
- Stain proteins on PVDF membrane by incubating the membrane in Ponceau red solution for 5 min (shaking)
- Remove excess dye by using Ponceau destaining solution for 5 min at rt (shaking)
- Wrap in e.g. saran wrap and scan blot to preserve the protein pattern; save as .tif-file
- Thereafter, wash membrane with PBS for 5 to 10 min at rt to completely remove Ponceau staining from blot

Blocking of unspecific binding sites on the membrane

- Incubate blots with blocking buffer for 1 h at rt (or on at 4° C) to prevent unspecific binding of antibodies to PVDF membranes

Optional: preincubation of a primary antibody (sc-13179) with a blocking peptide (sc-13179P)

- To test for specificity of the primary antibody sc-13179, the antibody was preabsorbed with a blocking peptide corresponding to a part of the MT1 sequence.
- Two tubes (1.5 ml) were prepared, each containing 10 µl of sc-13179 antibody (i.e. 2 µg antibody).
- A five-fold amount of blocking peptide sc-13179 (i.e. 10 µg, 50 µl) was added to one tube (tube B), while the other tube received 50 µl PBS only (tube A).
- PBS was added to both tubes to a final volume of 500 µl and tubes were incubated for 2 h at rt resulting in pre-absorption of the antibody in tube B.
- Thereafter, 1.5 ml blocking buffer was added to each tube and the two antibody solutions were added to two otherwise identically prepared blots. These blots were further processed as detailed in (see 5.4.6).

Antibody binding

- For each incubation step, 2 ml antibody solution per blot were prepared at desired dilution (table 5.1)
- Each blot was sealed in a plastic bag to minimize antibody consumption
- Blots were incubated with first antibody on at 4 °C (or 1 h rt), shaking; those (parts of the) blots receiving only secondary antibody were mean while kept in blocking buffer
- Wash blots at rt with PBS containing 0.1 % (v/v) Tween 20, change buffer at least 3 times and do each washing step for at least 15 min
- Incubate blots for 1 h at rt (or at 4° C) with HRP conjugated, secondary antibodies at desired concentration (table 5.1)

- Wash blots at rt with washing buffer, change buffer at least 3 times and do each washing step for at least 15 min

Detection of HRP-labelled antibody-complexes bound to membranes

- Detection is based on chemoluminescence.
- Mix solution A + B according to instructions of the manufacturer (final volume 0.1 ml / cm² or 2 ml / blot)
- Drain the excess wash buffer from the washed membranes
- Incubate surface of each membrane with 2 ml detection reagent for 5 min at rt
- Drain off excess detection reagent by holding blot against a paper towel
- Place the blot in a fresh piece of saran wrap and gently smooth out any air bubbles
- Place the wrapped blots (protein side up) in an x-ray film cassette
- Place a sheet of Hyperfilm ECL (adjust size!) on top of the membrane
- Close the cassette and expose for a certain time (e.g. 5 sec)
- Remove and develop the first piece of the film immediately and on the basis of its appearance estimate how long to continue the exposure of the second piece of film (this can vary from a few seconds to 1 h)
- Scan the developed films and store data as .tif-files

5.5 Immunofluorescence microscopy

5.5.1 Materials

- Cryotome (Microm HM500)
- Microscope (Zeiss Axiovert 200, Perkin Elmer Ultraview ERS or Zeiss Axioplan 2, Axiovision Software Rel 4.6)
- Centrifuges (Eppendorf 5415R; Rotixa/RP, Hettich)
- Waterbath (GFL, 1083), set to 50 °C
- Magnetic hot plate stirrer
- Thermometer (>65 °C)
- Multiwell Plates, tissue culture treated, sterile (Iwaki 3820 – 024)
- Cover slips, 12 mm diameter, thickness Nr. 1: 0,13 – 0,16 mm (Menzel-Gläser CB00120RA1)
- SuperFrost Microscope slides, ground edges 90° (Menzel - Gläser AG00008532E)
- Superfrost Plus Gold slides – for cryosections (Menzel - Gläser K5800AMNZ50)
- Parafilm M, (MENASHA, WI 54952)
- 10 ml single-use syringes (B. Braun, Melsungen AG H4606108V)
- Disposable sterile syringe filters, 25 mm, 0.20 Micron, cellulose acetate membrane acrylic (IWAKI 2052 – 025)

- Scissors and forceps
- Ice bucket
- Saran wrap
- Gloves
- Dako Pen (Dako S2002)

5.5.2 Reagents

- Paraformaldehyde (Merck 4005.1000)
- Ammonium chloride (Merck 1.01142.1000)
- Bovine serum albumin (BSA), Fraction V (PanBiotech P06 – 1391500)
- Goat serum (Life-Technologies PL16210064)
- DRAQ5 (Alexis BOS - 889 – 001 - R200)
- 4',6' Diamidino -2 phenylindol, DAPI (Roche 10236276001 Hana)
- Saponin (Sigma S - 1252)
- Glycerol, about 87 % (Merck 1.04094.0500)
- Mowiol 4 – 88 (polyvinylalcohol 4 – 88; FLUKA 81381)
- Tris (hydroxymethyl) aminoethane (Merck 1.08382.1000)
- Hydrochloric acid, fuming 37 % (Merck 1.00314.1000)
- Methanol (Fisher Scientific M/4000/17)
- Sodium azide (Sigma S 8032)
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck 1.02382.1000)
- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Merck 1.05886.1000)
- Sucrose (Merck 1.07653.1000)
- TissueTek O.C.T. COMPOUND (Sakura Finetek 4583)
- Primary and secondary antibodies (see Table 5.4)

Table 5.4 Primary and secondary antibodies (ABs) used to detect MT-1 by immunofluorescence microscopy

	Primary antibody	Secondary antibody
Antibody set I	Anti-Melatonin receptor 1A, affinity purified polyclonal AB, Raised in rabbit against a peptide representing an epitope in the 2nd cytoplasmic loop of MT1 Abcam, ab13035 Dilution 1:50 (200 µg / µl) and 1:100 (100 µg / µl)	Anti-rabbit IgG, Alexa Fluor® 568 conjugated AB produced in donkey mixed 1:1 with glycerol Invitrogen, A10042, Dilution 1:500
Antibody set II	Anti-Melatonin receptor 1A, affinity purified polyclonal AB, Raised in rabbit against a peptide that represents an epitope in the 3rd cytoplasmic loop of MT1 Abcam, ab13036 Dilution 1:50 (200 µg / µl) and 1:100 (100 µg / µl)	Anti-rabbit IgG, Alexa Fluor® 568 conjugated AB produced in donkey mixed 1:1 with glycerol Invitrogen, A10042, Dilution 1:500
Antibody set III	Anti - Melatonin receptor 1A, affinity purified polyclonal AB, Raised in goat against an N-terminal peptide of MT-1 Santa Cruz, sc-13179 Dilution 1:50 (4 µg / µl)	Anti-goat IgG, Alexa Fluor® 568 conjugated AB Produced in donkey mixed 1:1 with glycerol Invitrogen, A11057 Dilution 1:500
Antibody set IV	Anti - HAtag monoclonal AB, Produced in mouse GeneTex GTX21424 Dilution 1:100 (100 µg / µl)	Anti-mouse IgG, Alexa Fluor® 488 conjugated AB Produced in donkey mixed 1:1 with glycerol Invitrogen, A11001 Dilution 1:500

5.5.3 Buffers

PBS (See 5.1.4)

Culture medium for BeWo cell (See 5.1.4)

PBS containing 1 mM CaCl_2 and 0.5 mM MgCl_2 (PBS^{++} , sterile) (See 5.1.4)

1.1M sucrose solution

- Dissolve 18.8 g sucrose in 50 ml A.bid
- Keep at 4 °C, use fresh

4 % Paraformaldehyde Fixative (PFA/PBS)

- Add 4 g of Paraformaldehyde to 100 ml PBS
- Heat to 65 °C, stir in solution
- If necessary, add small portions of 1M NaOH for better dissolving of the PFA
- Gravity filter through a fine pore filter
- Store at 4 °C and use immediately
- After natively prepare (500 ml), aliquot (e.g. 50 ml) and freeze at -20 °C

50 mM ammonium chloride in PBS (50 mM NH_4Cl / PBS)

- Weigh in 1.34 g ammonium chloride
- Add 450 ml PBS
- stir in solution
- Bring to a final volume of 500 ml with PBS
- Store at rt

Blocking buffer (PBS containing 1 % (w/v) BSA and 0.05 % (w/v) saponin)

- Prepare a saponin stock solution of 10 % (w/v) saponin in PBS (e.g. 2 g saponin in 20 ml PBS), aliquot this stock and store at -20 °C
- Dissolve 5 g BSA in 450 ml PBS by stirring and add 2.5 ml of the saponin stock solution
- Bring to a final volume of 500 ml with PBS
- Aliquote (e.g. 50 ml) and store at -20 °C

Mowiol 4 - 88- embedding solution

- Put 6 g glycerol in a 50 ml tube
- Add 2.4 g Mowiol 4 – 88, dissolve with frequent agitation for 1 h at rt
- Add 6 ml A.bid., vortex and shake/stir for 1 h at rt
- Add 12 ml 0.2 M Tris/HCl, pH 8.5 + 0.02 % (w/v) NaN_3
- Incubate at 50 °C in a waterbath for up to 2 h (ev ery 20 min stir for 2 min)

- Clarify by centrifugation at 5000 x *g* for 15 min (usually, Mowiol 4 – 88 does not dissolve completely)
- Optional: Filter through a 0.2 µm syringe filter
- Aliquot (1 ml) and store at 4 °C

DRAG5 (Alexis Biochemical's)

- Store 5 mM stock at 4 °C

5.5.4 Preparation of coverslips for fluorescence microscopy

- Coverslips should be acid-washed to remove spots of dirt or detergent
- Place the coverslips in a glass beaker
- Cover with concentrated nitric acid
- Swirl for 5 min
- Discard the acid
- Rinse with A.bid.
- Rinse once with MeOH
- Sterilize the coverslips by baking for 4h at 80 °C

5.5.5 Seeding of cells on coverslips

Comment: Most cells (including BeWo cells) are fine plated directly onto glass. During plating these coverslips will become coated with vitronectin from the serum of the media. Some cells, however, will survive processing for IF much better if the coverslips are coated with specific substances before the cells are plated. Some cells prefer plastic supports.

- Add 12 mm sterile glass coverslips to wells of sterile 24 - well plates
- Add 1 ml of respective medium required for cultivation of cell line of interest
- Add detached cells (see 5.1.7) to wells using 1– 2 drops of a 10 ml cell suspension derived from a 75 cm² plate
- Grow cells for 2 days

5.5.6 Immunofluorescence staining of tissue culture cells

- Wash twice with 2 ml PBS (4 °C) / well
- Fix cells using 4 % PFA/PBS for 30 min at rt. Add 500 µl / well
- Wash cells 1 x with PBS
- Add 1 ml 50 mM NH₄Cl/PBS/well to quench any remaining PFA
- Block and permeabilize cells with 500 µl PBS containing 1 % BSA and 0.05 % saponin for 1 h at rt

- Incubate 1 h at rt with 20 µl diluted primary antibody (dilution in blocking buffer as indicated in table 5.4). To do incubation in such a small volume, place a of parafilm in a wet chamber, place the diluted antibody - solution on the parafilm and invert coverslip on the drop; following the incubation, return the coverslip to the well plate
- Note: Centrifuge all antibody - dilutions prior to their application at 12.000 x g 10 min at 4 °C to remove any aggregates
- As a control for unspecific binding, omit 1st antibody, using only secondary.
- Wash 3 times with PBS (each washing step at least 10 min)
- Incubate 1 h with secondary antibody at rt in the dark (diluted in blocking buffer as indicated in table 5.4)
- Wash cells 3x with PBS (each washing step at least 10 min)
- Finally die glass slid in A.bid. and remove excess water by dipping again a slip briefly paper Towel
- Put a drop of Mowiol 4 – 88 solutions (10 µl) on a glass slide and embed inverted coverslip in Mowiol. Allow Mowiol to dry over night in dark
- View samples using a confocal fluorescence microscope (Zeiss Axiovert 200 + Perkin Elmer Ultraview ERS)
- Process images by using Zeiss Axiovision software and Adobe Photoshop software.

5.5.7 Collection and preparation of placental tissue for cryo sectioning

- Pieces of placental Tissue are prepared as indicated in (see 5.1.5), washed 5 times in PBS++ on ice and cut it in smaller pieces (1 – 3 mm²)
- Fix the tissue in 4 % PFA/PBS for at least 4 h to on at rt
- Wash tissue for 10 min in PBS and transfer into 1.1 M sucrose solution and incubate on at 4 °C
- Cover 12 or 24 well plate with a sheet of saran wrap large enough to fill all holes and fill the wells (according to the size of the tissue) with Tissue Tek
- Dip tissue briefly into water
- Remove excess fluid by briefly dipping the tissue against a paper towel
- Transfer single pieces of tissue to wells containing Tissue Tek
- Freeze samples in plate at -80 °C
- Next day, transfer the Tissue Tek-blocks containing tissue from the plate to labelled storage containers at -80 °C

5.5.8 Preparation of placental cryosections

Comment: Frozen placental sections are prepared on a cryotome, which consists of a movable holder for the tissue, and a fixed knife. The cryotome is kept at -20 °C, the knife and the sample holder all cooled down to between -22° to -25 °C (temperature settings may vary with type of cryotome, but it seems to be useful that the knife and the holder are cooler than the surrounding).

- To mount the tissue block on the specimen holder, add some TissueTek on top of the sample holder, and as it starts to freeze, pop out the frozen tissue block and dip into TissueTek.
- Mount the specimen in the machine, and start sectioning (20 or 25 µm sections) thereby trimming until you see tissue. If the sections do not flatten or have cracks/splitting, adjust the:
 - o Angle of sectioning (for placental tissue a flat angle seems fine), or
 - o speed of movement (cut sections manually - slow but with constant pressure), or
 - o position of the knife (you may have a crack in the knife edge), or
 - o the position of the anti-roll plate (it works fine, if the anti-roll plate overlaps the edge of the knife for approx. 0.5 to 1 mm).
- Adjust the desired section thickness (4 – 5 µm) and start sectioning
- Between sections, clean the knife from all tissue traces with acetone and trim the sample several times until it is plane again
- Position 2 – 3 sections on a slide (Superfrost Plus Gold) by moving the slide down onto the section lying on the knife. It will attach immediately to the slide.
- Label your slides as you prefer, but at least with a section number, and ID of the tissue block.
- If cryosections are not immediately used for staining: allow them to air dry
- Store the sections at -20 °C (for longer storage use -80 °C). Put the slides together in aluminium foil (always two together, back to back) and label them. Alternatively the slides can be stored inside a zip-lock bag containing dessicant.

5.5.9 Immunofluorescence staining on frozen tissue section

- Block and permeabilize cells with PBS containing 1 % BSA and 0.05 % saponin for >30 min at rt
- Incubate 1 h with primary antibody (diluted in PBS containing 1 % BSA and 0.05 % saponin) at rt
- Wash 3 times with PBS (allow at least 8 – 10 min per washing step)

- Incubate 1h with secondary antibody (diluted in PBS containing 1 % BSA and 0.05 % saponin at rt in the dark)
- Wash 3 times with PBS (allow at least 8 – 10 min per washing step)
- Use DRAQ5 (diluted 1:1000; 5 μ M) to stain nuclei, incubate for 5 min in the dark
- Wash 5 times with PBS (allow at least 8 – 10 min per washing step)
- Dip glass slide in A.bid. and remove excess water
- Embed in Mowiol - solution and cover the sections with a glass coverslip
- Allow Mowiol to dry in the dark
- View samples using a confocal fluorescence microscope (Zeiss Axiovert 200 + Perkin Elmer Ultraview ERS)
- Process images by using Zeiss Axiovision software and Adobe Photoshop software.

5.6 Transformation of competent bacteria (E.coli)

5.6.1 General remarks

A **plasmid** is a small circular piece of DNA (about 2,000 to 10,000 base pairs). Although many different kinds of plasmids are available, all of them contain the following elements. 1/ A selectable marker, that is a gene encoding for an antibiotic resistance. 2/ A origin of replication, which is used by the DNA making machinery in the bacteria as the starting point to make a copy of the plasmid. 3/ A multiple cloning site, that contains many restriction enzyme sites and is used to insert the DNA of interest. Following insertion of the gene of interest into the plasmid, these plasmids are amplified in bacteria.

Transformation of bacteria [396]: a foreign plasmid is introduced into bacteria and those bacteria are used to amplify the plasmid in order to make large quantities of it. As plasmids usually contain a resistance against an antibiotic (e.g. ampicillin), growing the transformed bacteria in ampicillin provides a selective pressure, that allows only bacteria that have acquired the plasmid to grow. Therefore, as long as bacteria are grown in the antibiotic, the plasmid is required to survive and will be continually replicated, along with the gene of interest that has been inserted to the plasmid.

Competent Cells: Since DNA is a very hydrophilic molecule; it won't normally pass through a bacterial cell's membrane. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA. This is done by creating small holes in the bacterial cell membrane by suspending them in a solution with a high concentration of calcium. DNA can then be forced into the cells by incubating the cells and the DNA together on ice, placing them briefly at 42 °C (heat shock), and then putting them back on ice.

5.6.2 Materials

- High-speed Centrifuge (RC5C Sorvall Instrument) with rotor SS34 or F21-S
- Incubator shaker (Series 25D, new Brunswick Scient. Co., Inc.), set to 37 °C and 300 rpm
- Waterbath, adjusted to 42 °C
- Reaction tubes, 1.6 ml (Biozyme 710160), sterile
- 90 mm Petri dishes (Sterilin 101RT)
- Drigalski spatula
- 10 ml single-use syringes (B. Braun Melsungen AG H4606108V)
- Disposable sterile syringe filters, 25 mm, 0.20 Micron, cellulose acetate membrane acrylic (IWAKI 2052 – 025)
- 15 ml tube (Falcon 2059)
- Tooth pick , autoclaved

5.6.3 Reagents

- E. coli (Strain JM107, M0109 Fermentas)
- Bacto-Trypton (Invitrogen Q100 – 29)
- Bacto-Yeast Extract (Merck, 1.03753)
- Bacto-agar (Calbiochem 12177)
- Ampicillin, sodium salt (Amresco 0339)
- NaCl Merck (1.06404.1000)
- KCl Merck (4936.1000)
- NaOH (1.06498.1000)
- MgCl₂ (anhydrous, Merck 5832.500)
- CaCl₂ (anhydrous, Merck 2383.500)
- Glucose (Merck 3075.0500)
- Glycerol, 87 % (Merck 1.04094.0500)

5.6.4 The plasmid

The plasmid *mel1a-3xHA-pcDNA3* (20 µl containing 400 µg / µl) was obtained from Dr. Tarja Kokkola, (Institute of Human Genetics; University Medical Center Hamburg-Eppendorf; Hamburg; Germany). The plasmid is composed of pcDNA3 (Invitrogen; see Figure 5.1) containing the coding region of the human MT1 melatonin receptor cDNA [397, 398]. To allow for the detection of receptor protein in immunological assays, a triple hemagglutinin (HA) epitope tag was subcloned after the initiating Met codon of the MT1 receptor. Triple HA tag contains three nine-amino acid sequences (YPYDVPDYA)

pCDNA3 is a mammalian expression vector designed for high-level stable and transient expression in mammalian host. The vector contains a human cytomegalovirus immediate-early (CMV) promoter for high level expression in a wide range of mammalian cells, multiple cloning sites to facilitate cloning and a neomycin resistance gene for selection of stable cell lines

250 mM KCl

- Dissolve 20 g NaOH in a final volume of 100 ml A. bid.
- Store at rt

- Dissolve 19 g MgCl_2 in a final volume of 100 ml A. bid.
- Autoclave
- Store at rt

1 M glucose

- Dissolve 18 g glucose in a final volume of 100 ml A. bid.
- Filter sterilize through a 0.22 μm filter
- Store at 4 $^{\circ}\text{C}$

1M CaCl_2

- Dissolve 11.1 g of CaCl_2 (anhydrous) in a final volume of 100 ml A. bid.
- Sterilize by filtration (sterile syringe filters, 0.20 Micron).
- Store at 4 $^{\circ}\text{C}$

0.1M CaCl_2

- Mix 50 ml of sterile 1M CaCl_2 with 450 ml of sterile A.bid.
- Store at 4 $^{\circ}\text{C}$

50 % glycerol

- Mix 50 ml of glycerol with 50 ml of A. bid.
- Autoclave
- Store at rt

0.1M CaCl_2 + 20 % glycerol:

- Mix 10 ml of sterile 1M CaCl_2 , 40 ml of sterile 50 % glycerol, and 50 ml of sterile A.bid.

SOC medium, sterile - stock solution

SOC Medium is a rich medium used primarily to aid recovery of bacterial competent cells following transformation. Use of SOC medium improves the molecular uptake whilst stabilizing the cells rapidly and so maximizing the efficiency of competent cells.

- 20 g Bacto-Trypton (final concentration 2 % w/v)
- 5 g Bacto-Yeast Extract (final concentration 0.5 % w/v)
- 0.6 g NaCl (final concentration 10 mM)
- Dissolve in 950 ml A. bid.
- Add 10 ml of 250 mM KCl (final concentration 2.5 mM)
- Adjust pH with 5 N NaOH (~0.2 ml) to 7.0
- Adjust to 1 l with A.bid.
- Autoclave
- Store at 4 $^{\circ}\text{C}$

SOC medium, sterile-ready-to-use solution

- Before use add - under sterile conditions
- 5 ml of sterile 2M MgCl_2 (final concentration 10 mM)
- 20 ml of 1 M glucose (final concentration 20 mM)
- If not used immediately This solution should be stored in aliquots at -20 °C

LB (Lysogeny Broth) Medium

- 10 g Bacto tryptone (final concentration 1 % (w/v))
- 5 g Bacto-yeast extracts (final concentration 0.5 % (w/v))
- 10 g NaCl (final concentration 1 % (w/v))
- Dissolve in 950 ml A.bid.
- Adjust the pH with 5 N NaOH (~0.2 ml) to pH 7.0 and the volume to 1 l
- Sterilize by autoclaving
- Store at 4 °C

LB Plates

(Stocks are stored in the 4 °C room in the bacteri a refried.)

- Prepare LB medium
- Add 15 g / liter bacto-agar (final concentration 1.5 % (w/v))
- Autoclave
- Mix gentle after removing from autoclave
- Cool to ~50 °C before adding antibiotic
- Add ampicillin to a final concentration of 50 µg / ml (dilute 1:1000 from a 50 mg / ml stock)
- Pour plates (~30 – 35 ml agar / 90 mm dish)
- Flame the surface with a Bunsen Brenner before hardened to avoid air bubbles
- When hardened completely, invert plates, return in the original plastic back, seal it and store at 4 °C.

Ampicillin stock solution (1000 x)

- Dissolve Ampicillin at a concentration of 50 mg / ml A.bid
- Prepare e.g. 25 ml and aliquote in 1 ml portions
- Store at -20 °C

5.6.6 Preparation of CaCl_2 -competent *E. coli* cells

- Perform whole procedure on ice and under sterile conditions
- NOTE: through the process, cells should be treated with care. No vortexing or excess pipetting should be performed, especially when the cells have been resuspended in CaCl_2 because lysis will result, decreasing the amount of competent cells.
- Prepare an overnight culture of *E. coli* (Strain JM107) in 5 – 10 ml LB medium, keep an aliquote for OD measurement
- Inoculate 50 ml LB medium with the overnight culture (dilute on culture 1:100) and shake for 2 h at 37 °C (OD 600 around 0.34)
- Place on ice for 10 – 20 min
- Centrifuge for 10 min at 5500 rpm (SS34 or equivalent) at 4 °C
- Remove the medium completely
- Resuspend the pellet in 25 ml ice-cold 0.1 M CaCl_2
- Incubate for 30 min on ice in the cold room
- Centrifuge for 10 min at 5500 rpm (SS34) at 4 °C
- Remove the supernatant thoroughly
- Resuspend the pellet in 5 ml ice-cold 0.1 M CaCl_2 / 20 % glycerol
- Shockfreeze the competent cells in liquid nitrogen and store aliquots (50 – 100 µl) at -80 °C

5.6.7 Transformation of competent bacteria with plasmid mel1a-3xHA-pcDNA3

- Thaw competent bacteria (50 µl portion) the cells on ice, this is important because the bacteria are very sensitive when competent
- Add to each tube ~1 – 2 µl of plasmid DNA (should not be more than 50 µg)
- Mix by tapping - do not vortex or mix by pipetting
- Incubate 30 min on ice
- Incubate cells for 30 sec at 42 °C (exactly)
- Place rapidly on ice, and keep there for 1 – 2 min
- Prepare 250 µl of pre-warmed SOC medium (WITHOUT antibiotics) in new vials (aliquot SOC from stock under sterile conditions, otherwise SOC medium gets contaminated),
- Add bacteria and incubate at 37 °C for 1 h, shaking at 300 rpm.
- Meanwhile, transfer LB-plates + ampicillin (stored at 4 °C) to rt ~60 min before using them (agar side up) and remove any water from the lid before inverting them
- Transfer 20 and 200 µl respectively each transformed bacteria solution to the plates

- Distribute evenly on surface using the Drigalski spatula and keep at rt until liquid is absorbed (the remaining transformation mix may be stored at 4 °C and spread out on a plate the next day if problems arose)
- Invert plates (agar side up) and incubate them at 37 °C for 12 – 16 h; colonies should appear then
- Be aware that ampicillin plates should not be incubated longer than 20 h, β -lactamase secreted by amp-resistant bacteria rapidly inactivates the antibiotic in the agar surrounding the bacteria, therefore satellite colonies may form that may not contain plasmid
- Store plates inverted at 4 °C if necessary, be aware that viability of bacteria at 4 °C is limited.

5.6.8 Long-time storage of transformed bacteria

Plates with transformed colonies of bacteria can be stored for several weeks, up to several months; however the colonies lose their viability, therefore for long-time storage of transformed bacteria prepare glycerol stocks.

- Inoculate 1.5 – 2 ml of LB medium containing the appropriate antibiotics (50 µg / ml ampicillin) in a ~15 ml tube with a toothpick (autoclaved) from a single colony. Pick transparent (=viable) colonies from the plates, prepare ~12 colonies
- Incubate for at least 6 h at 37 °C with vigorous shaking, but better allow growing overnight.
- Take an 0.8 ml aliquot from each tube and add 0.2 ml sterile glycerol to each tube, mix and freeze cells at -80 °C
- For bacterial recovery plate the bacteria on an appropriate LB-agar plate (containing antibiotic)

5.7 DNA Purification Protocols

5.7.1 Materials

- Microcentrifuge (Eppendorf, 5415D), cooled to 4 °C (in cool-room) and
- Mini - Vac Power (peQlab Biotechnologie GmbH)
- Incubator shaker (Series 25D, New Brunswick Scient. Co., Inc.), set to 37 °C and 300 rpm
- 15 ml tube, sterile
- Reaction tubes, 1.6 ml (Biozyme 710160), sterile
- 10 ml single-use syringes (B. Braun Melsungen AG H4606108V)
- Disposable sterile syringe filters, 25 mm, 0.20 Micron, cellulose acetate membrane acrylic (IWAKI 2052 – 025)

- Toothpicks, sterile

5.7.2 Reagents

- Bacto-Trypton (Invitrogen Q100 – 29, 500 g)
- Bacto-Yeast Extract (Merck, 1.03753, 500 g)
- Bacto-agar (Calbiochem 12177, 250 g)
- Ampicillin, sodium salt (Amresco 0339, 25 g)
- NaCl (Merck 1.06404.1000)
- NaOH (Merck 1.06498.1000)
- Glucose (Merck 4074.0500)
- Tris (Hydroxymethyl aminoethan) (Merck 1.08382.1000)
- EDTA (Merck 1.08418.1000)
- Potassium acetate (Merck 4820)
- Glacial acetic acid (Merck 8.18755.2500)
- 100 % ethanol (Merck 1.00983 – 1000)
- 70 % ethanol
- Promega Pure Yield Plasmid Midiprep System

5.7.3 Buffers

5 N NaOH

- Dissolve 20 g NaOH in a final volume of 100 ml A. bid.
- Store at rt

(Lysogeny broth) Medium

- 10 g Bacto tryptone
- 5 g Bacto-yeast extracts
- 10 g NaCl
- Dissolve in 950 ml A.bid.
- Adjust the pH with 5 N NaOH (~0.2 ml) to pH 7.0 and the volume to 1 l
- Sterilize by autoclaving
- Store at 4 °C

Ampicillin stock solution (1000 x)

- Dissolve Ampicillin at a concentration of 50 mg / ml A.bid
- Prepare e.g. 25 ml and aliquote in 1 ml portions
- Store at -20 °C

Solution I

- 4.50 g glucose (final concentration 50 mM)
- 1.97 g Tris-Cl (final concentration 25 mM)
- 1.86 g EDTA (final concentration 10 mM)
- Dissolve reagents in 300 ml A.bid.
- Adjust pH to 8.0 with HCl.
- Adjust volume to 500 ml.
- Aliquote (100 ml)
- Sterilize by autoclaving.
- Store at 4°C
- Note: Before use, add RNase A (final concentration 0.1 g / l or 10 mg / 100 ml) from 1 ml stock aliquots of RNase A at 10 mg / ml, stored at -20 °C.

Solution II

- To 86 ml A.bid.
- Add 10 ml 10 % SDS (final concentration 1 %)
- And 4 ml 5 M NaOH (final concentration 200 mM). Do not add the SDS directly to the NaOH as a precipitate will form
- no need to sterilize, do not autoclave
- Prepare fresh, keep at rt

Solution III

- Dissolve 29.45 g of potassium acetate in 50 ml A.bid.
- Add 11.5 ml glacial acetic acid
- Bring up to a final volume of 100 ml
- The final solution is 3 M potassium and 5 M acetate
- The pH should be 5.5
- Autoclave if sterile reagents are not used
- Store at 4 °C.

5.7.4 Alkaline Lysis

Alkaline lysis was first described by [399] and with a few modifications it is the preferred method for plasmid DNA extraction from bacteria ever since. The below protocol is seized for Minipreps and is a tool to quickly purify plasmid DNA from a series of different clones of transformed bacteria for further analysis of the plasmid (restriction enzyme digestion, PCR reactions,...)

- Inoculate 2 ml of LB medium containing the appropriate antibiotics (50 µg / ml ampicillin) in a ~15 ml tube with a toothpick from a single colony. Pick transparent (=viable) colonies from the plates, prepare ~12 colonies
- Incubate for at least 6 h at 37 °C with vigorous shaking, but better allow to grow overnight (colonies were grown on).
- Use 1.5 ml of the culture for a miniprep (alkaline lysis), store the remainder at 4 °C (maybe you will need it to inoculate another culture)
- Centrifuge 1.5 ml of miniprep for 5 min in Eppendorf centrifuge (14.000 x g), discard supernatant by aspiration. The supernatant can be conveniently removed with a disposable pipette tip attached to a vacuum line. Use a gentle vacuum and touch the tip to the surface of the liquid. Keep the tip as far away from the bacterial pellet as possible as the fluid is withdrawn from the tube. The pipette tip can then be used to vacuum the walls of the tube to remove and adherent droplets of fluid.
- Resuspend pellet completely by pipetting up and down in 100 µl of ice cold solution I
- Add 200 µl of solution II and mix by inverting the tube several times (do not vortex!), keep on ice. Alkaline-SDS lysis must be done gently to avoid contamination with chromosomal DNA. The SDS lyses the cells and the NaOH denatures proteins and chromosomal (linear) DNA. Optimal pH of mixture at this point is between 12.0 and 12.6.
- Add 150 µl of solution III and vortex inverted tube for 10 sec, leave on ice for 3 – 5 min. Addition of solution III readjusts pH to neutral (solution III contains acetic acid) and its high salt content (3 M in potassium acetate) precipitates SDS-protein complexes, RNA, and chromosomal DNA.
- Spin 5 min (14.000 x g), this will give a big white pellet, transfer the supernatant in a new Eppendorf tube and repeat the spin
- Transfer the sup in a new tube, precipitate plasmid DNA with 1 ml (96 %) Ethanol (2 volumes)
- incubate at -20 °C for 10 min
- spin for 10 – 15 min in Eppendorf centrifuge (14.000 x g)
- Discard sup and wash pellet with 500 µl of 70 % Ethanol by vortexing
- spin to collect pellet again
- Discard sup, invert open tube on a paper towel, then remove remaining drops with a disposable tip attached to a vacuum line and dry pellet for ~10 min on bench
- Redissolve pellet in 50 µl A.bid. and store at -20 °C
- analyze by restriction enzyme digestion

5.7.5 DNA (Plasmid) purification

- Prepare 100 ml LB-Medium containing antibiotics (50 µg / ml ampicillin) in a sterile conical (Erlenmeyer) flask
- Inoculate this medium with 10 µl Bacteria suspension
- Shake on at 37 °C and 325 rpm ($OD_{600} = 2 - 4$)
- Pellet cells at 5000 x g for 10 min and discard supernatant
- Purify plasmid DNA according to the protocol of the manufacturer
- Finally, following elution from the column, resuspend DNA in 400 µl A.bid.
- Determine concentration and purity of plasmid DNA according to (see 5.8)

5.8 DNA quantitation and purity determination

The concentration of DNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per ml. This is valid for measurements in water. A ratio of readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of DNA with respect to contaminants that adsorb in the UV, such as proteins.

- To determine the DNA content, mix 398 µl DEPC treated water with 2 µl of the DNA solution
- After vortexing the samples, measure the absorption in a spectrophotometer at 260 nm and 280 nm (re-zero the spectrophotometer using water)
- Multiplicating the A_{260} value with the factor of 8 results in the amount of µg DNA in 1 µl water (µg / µl) ($[DNA] = \text{DNA concentration, dilution factor} = 200$)

$$[DNA] = \frac{50 * A_{260} * \text{dilutionfactor}}{1000} = \mu\text{g} / \mu\text{l}$$

- The A_{260}/A_{280} ratio can be used as an indicator for RNA purity and it should be > 1.8

5.9 Restriction Enzyme digestion of plasmid DNA (*mel1a-3xHA-pcDNA3*)

5.9.1 General remarks

Restriction enzymes are enzymes isolated from bacteria that recognize specific sequences in DNA and then cut the DNA to produce fragments, called restriction fragments. If plasmids of known origin are used, this is a simple method to control whether the right plasmid is handled. Digestion with the restriction enzymes should give a characteristic pattern of bands following analysis by native agarose gel electrophoresis. However, other methods should be used to exclude any mutations (e.g. sequencing).

With respect to *mel1a-3xHA-pcDNA3*, the pcDNA3 plasmid has a size of 5.5 kB, the MT1 insert is 1.1 kb in size. Based on the sequence analysis of pcDNA3 and MT1,

digestions with either Hind III alone or combined digestion using EcoRI + XhoI or XbaI or ApaI will give respective DNA fragments.

5.9.2 Materials

- Waterbath adjusted to 37 °C

5.9.3 Reagents

- Restriction enzyme HindII (Promega R6041), supplied with buffers and acetylated BSA

5.9.4 Digestion with restriction enzymes

- For the restriction digestion mix the following in an Eppendorf tube (autoclaved):
 - o 1 µl plasmid (~0.1 – 0.5 µg DNA)
 - o 0.5 µl restriction enzyme (Hind III, Promega)
 - o 1 µl 10 x RE buffer (Buffer E, Promega)
 - o 10 µl acetylated BSA (1:10 dilution of the stock), Promega
 - o Fill up to 10 µl with A.bid.
- Incubate at 37 °C for 1h – 1h 30 min, stop incubation by placing tubes on ice.
- Analyse fragments by native gel electrophoresis (see 5.11)

5.10 Polymerase chain reaction (PCR) for verification of a plasmid sequence

5.10.1 General remarks

Polymerase chain reaction (PCR) is a technique to exponentially amplify *in vitro* a small quantity of a specific nucleotide sequence. This requires the presence of a template sequence, two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA and a thermo stable (taq) DNA polymerase in an appropriate buffer. The reaction is cycled involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase until enough copies are made for further analysis (e.g. native gel electrophoresis).

5.10.2 Materials

- Personal Cyclor (Biometra)
- PCR tubes (Soft strips and Cap strips, DNA, DNase, RNase and PCR-Inhibitor-free, Biozyme 711630 and 711640)
- Safe-Seal Tips (PeqLab, e.g. 20 µl # 81 – 1020, 200 µl # 81 – 1040, 1000 µl # 81 – 1050)

5.10.3 Reagents

- cDNA, stored at -20 °C
- Ultrapure DNase/RNase free water (Invitrogen 10977035)
- Taq DNA Polymerase (1 U / µl; Fermentas EP0404), stored at -20 °C
- 10x Taq DNA Polymerase (Fermentas EP0404), stored at -20 °C
- 25 mM MgCl₂ (Fermentas EP0404), stored at -20 °C
- 10 mM (each) dNTP mix (Fermentas EP0404), stored at -20 °C
- Loading dye solution (Fermentas R0611)
- Marker: Fermentas Gene Ruler 100 bp marker (Fermentas SM0241).
- Sense and antisense primers to amplify MT1 (1 µM each), stored at -20 °C

MT1 sense primer [400]: 5'-TCCTGGTCATCCTGTCGGTGTATC-3'

MT1 antisense primer [400]: 5'-CTGCTGTACAGTTTGTCTACTTG-3'

5.10.4 PCR Mastermix for 1 sample

(final volume/sample = 24 µl)

- | | |
|-----------------------------|---------|
| • 10x Taq DNA Polymerase | 2.5 µl |
| • MgCl ₂ (25 mM) | 2.0 µl |
| • dNTP mix (10 mM each) | 0.5 µl |
| • primer sense (1 µM) | 0.25 µl |
| • Primer antisense (1 µM) | 0.25 µl |
| • Water | 17.5 µl |
| • Taq Polymerase (1 U / µl) | 1.0 µl |

5.10.5 Protocol for PCR reaction

- Dilute the plasmid DNA 1: 200 in nuclease free water
- take 1 µl of this dilution for the PCR reaction
- Prepare the PCR Mastermix in a 1.5 ml reaction tube
- Calculate the volumes according to the number of samples (n) being tested and add 5 % excess volume,
*Formula: $[(n + 1) * 1.05] * (\text{vol for single reaction}) = \text{needed volume } [\mu\text{l}]$ e.g the amount of 10x buffer for 10 samples would be 28.9 µl*
- Distribute 24 µl of PCR mastermix for each sample to a 0.2 ml PCR strip tube
- Add 1 µl of template DNA.
- Run PCR
- PCR cycling program:

- | | |
|----------|-------|
| 1. 94 °C | 2 min |
| 2. 94 °C | 15 s |
| 3. 65 °C | 30 s |
| 4. 72 °C | 1 min |
| 5. 72 °C | 5 min |
| 6. 4 °C | ∞ |

- Repeat steps 2 – 4 x 30 times
- After the PCR run, analyse fragments by native agarose gel electrophoresis (see 5.11)

5.11 Native agarose gel electrophoresis

5.11.1 Background

Agarose gel electrophoresis is used to separate DNA or RNA molecules by size. The negatively charged nucleic acid molecules are moved through an agarose matrix with an electric field. Shorter molecules move faster. In native agarose gel electrophoresis the samples are not denatured; usually, different forms (conformations) of genetic molecules may run in unpredictable forms. To avoid this problem, linear molecules of DNA are usually separated. This type of electrophoresis is used to analyse the size of PCR products or the size of fragments generated by restriction-enzyme digestion.

Native agarose gel electrophoresis is sufficient to judge the integrity and overall quality of a total RNA preparation by inspection of the 28S and 18S rRNA bands. The secondary structure of RNA that is formed via intramolecular base pairing alters its migration pattern in native gels, therefore it will not migrate according to its true size; if this is a problem, a denaturing agarose gel should be applied.

Intact total RNA will have a major 28S band that should be approximately twice as intense as the 18S band. Completely degraded RNA will appear as a very low molecular weight smear.

Ethidium bromide (EtBr) intercalates with DNA and RNA resulting in fluorescence under UV light. By running nucleic acids through an EtBr treated gel, distinct DNA/RNA bands can be visualized under UV light.

5.11.2 Materials

- Microwave oven
- Electrophoresis chamber (Perfect Blue MaxiS, PeQLab) including gel casting tray and combs
- Power supply (Consort EV231 PeQLab)
- Transilluminator (HeroLab)

5.11.3 Reagents

- Agarose D-12E (Margaritella 1015 – 1005)
- Tris (Hydroxymethyl aminoethan; Merck 1.08382.1000)
- Boric Acid (Promega Merck 1.00165.1000)
- Ethylenediamine tetraacetic acid (EDTA), disodium salt dihydrate (Amresco 0105 – 500g)
- Bromphenolblue (Amresco 0449 – 25G)
- Xylene cyanol FF (Amresco 0819 – 20G)
- Glycerol (Merck 1.04094.0500)
- Ethidium bromide (Amersco 492 – 5g)
- Molecular weight markers: FastRuler DNA Ladder, High Range, ready-to-use, (Fermentas SM1128) and Fermentas O'GeneRuler Express DNA Ladder, ready-to-use (Fermentas SM1568)

5.11.4 Buffers

0.5 M EDTA stock-solution, pH 8.0

- Dissolve 93.05 g EDTA disodium salt in 400 ml A.bid.
- Adjust the pH with NaOH to about 8.0 (EDTA will not go completely into solution until the pH is adjusted to about 8.0)
- Bring to a final volume of 500 ml stock with A.bid.
- Store at rt

5x Tris-borate-EDTA (TBE) Running Buffer

- Dissolve 54 g Tris base
- and 27.5 g boric acid
- in approximately 900 ml A. bid.
- Add 20 ml of 0.5 M EDTA (pH 8.0)
- Adjust the solution to a final volume of 1 l.
- This solution can be stored at rt but a precipitate will form in older solutions. Store the buffer in glass bottles and discard if a precipitate has formed.

0, 5 x TBE Running Buffer

- To 100 ml of 5 x TBE stock solutions add 900 ml A.bid.
- store at rt

6x Loading Dye

- Dissolve 25 mg Bromphenolblue (0.25 % w/v),
- And 25 mg Xylene cyanol FF (0.25 % w/v)
- And 3 ml glycerol (30 % w/v)
- in a A. bid.
- Final volume 10 ml
- store in 1 ml aliquots at -20 °C, keep 1 aliquot at rt

Ethidium bromide (EtBr) stock-solution

- Prepare a 10 mg / ml solution in A.bid.
- store at rt
- EtBr is a known mutagen and should be handled as a hazardous chemical. Wear gloves while handling!

Preparation of agarose DNA gels

- Close the open ends of the gel casting tray(s) with tap, while the gel is being casted (remove the tap prior to electrophoresis)
- To pour a gel, mix agarose powder with TBE electrophoresis buffer to the desired concentration (indicated in the result section). For a 1 % gel, mix 0.3 g agarose with 30 ml 0.5 x TBE buffer. Higher concentration of agarose allow separation of small DNA, while low agarose concentrations allow resolution of large DNA)
- Heat this mixture in the microwave oven until completely melted (at 800 W for ~12 min)
- Cool the solution to about 60 °C
- Add 1.5 µl of EtBr stock solution (final concentration 0.5 µg / ml)
- Pour the gel into the casting tray containing a sample comb and allow to solidify at rt (~30 min)

5.11.5 Sample preparation

- PCR products: Mix 10 µl PCR product and 2 µl loading dye
- RNA samples: Mix the appropriate volume of 1 µg RNA (obtained after RNA isolation) with 2 µl loading dye and bring to a final volume of 12 µl with RNase free water
- RE-digestions: mix 10 µl digested plasmid and 2 µl loading dye

5.11.6 Native agarose gel electrophoresis

- DNA will move forwards the positive electrode. Bromphenol blue and Xylene Cyanol will migrate through agarose gels at roughly the same rate as double stranded DNA

fragments of 300 and 4000 bp, respectively. This can be used to judge the distance the DNA has migrated through the gel.

- Remove the comb and place gel in casting tray (tap removed!) in the submarine agarose gel unit
- Add 0.5x TBE buffer, the gel should be just covered
- Apply 10 µl samples mixed with loading dye to sample wells
- Apply 3 µl of DNA marker to a sample well
- Place lid and power leads on the apparatus and apply current
- Running conditions ~ 5 V/cm; cm = distance between the two electrodes)

5.11.7 Visualization of DNA/RNA fragments

- After adequate migration, the gel is placed on a UV transilluminator
- Examination and photography should take place shortly after cessation of electrophoresis, as the DNA diffuses in the gel over time
- In the case the EtBr staining is not intense enough; the gel can be stained after the electrophoresis by soaking in a dilute solution of EtBr.

5.12 Verification of sequence by automated sequencing

- The Plasmid was sent to DI Martin IBL (www.ibl.or.at) in a concentration of 0.5 µg / µl; at least 3 µg per run were required.
- Automated sequencing of the inserted DNA in pcDNA3 vector was performed with the BigDye Terminator-principle using an AB 3100 Genetic analyser (Applied Biosystems) in both directions using T7 and SP6 specific primers.

5.13 Transfection of eukaryotic cells

5.13.1 General remarks

The possibility to stably integrate genes into the genome of mammalian cells has an important impact on many biomedical research areas as well as for the development of pharmaceutical products. While transient transfection is advantageous for fast analysis of genes and small scale protein production, stable transfection ensures long-term, reproducible as well as defined gene expression. Stable expression is achieved by integration of the gene of interest into the target cell's chromosome: Initially the gene of interest has to be introduced into the cell, subsequently into the nucleus and finally it has to be integrated into chromosomal DNA.

Stable expression can be influenced by two factors: the vector containing the gene of interest and the transfection method used.

The type of vector used for stable integration defines the integration mechanism, the regulation of transgene expression and the selection conditions for stably expressing cells. After integration the level and time of expression of the gene of interest depends on the promoter cloned upstream on the expression vector and on the particular integration site. For constitutive expression, promoters such as the CMV promoter are chosen. Furthermore the site of integration can have an effect on the transcription rate of the gene of interest. Usually a regular expression plasmid is integrated into the genome of the target cell randomly. Integration into inactive heterochromatin results in little or no transgene expression, whereas integration into active euchromatin frequently allows transgene expression. However, random integration often leads to silencing of the transgene.

The exact mechanism by which plasmid DNA is integrated is not yet fully understood and remains a matter of research. In viral systems, the foreign DNA is integrated into the host genome via viral integration mechanisms. Plasmid DNA delivered by non-viral methods, on the other hand, is integrated by the cell's machinery itself, possibly via DNA repair and recombination enzymes.

Stably transfected cells can be selected and cultured in various ways: For the selection of stably transfected cells, a selection marker is co-expressed on either the same or on a second, co-transfected vector. A variety of systems for selecting transfected cells exists, including resistance to antibiotics such as neomycin phosphotransferase, conferring resistance to G418. The culture of the transfected cells can be done either in bulk to obtain a mixed population of resistant cells, or via single cell culture, to obtain cell clones from one single integration event.

Calcium phosphate transfection, initially described in the early 1960s, was refined and systematically improved to result in a standard protocol that has changed little since the early 1970s [401]. Nucleic acids alone cannot penetrate the cell membrane. By this technique precipitates of plasmid DNA are formed by its interaction with calcium ions; the DNA precipitates then enter the cell by endocytosis. It is a very inexpensive and simple technique to perform. Plasmid DNA is mixed in a solution of calcium chloride, and then is added to a phosphate- buffered solution. Over a period of 20 minutes a fine precipitate forms in the solution, and this solution is then added directly to the cells in culture. Transfer efficiency, the number of cells which express the desired gene, is usually quite limited and only reaches levels greater than 10 % in a few specific cell lines. In many cases, the level is less than 1 %. Transfection efficiencies can be improved in some cell lines by 'shocking' the cells with DMSO or glycerol. Cells can be either transiently or stably transfected using this technique.

5.13.2 Material

- See 5.1.2 (cell culture)
- Microcentrifuge (Eppendorf, 5415D)
- Reaction tubes, 1.6 ml (Biozyme 710160)
- 100 mm dishes (tissue-culture treated, 3020 – 100 Iwaki)
- 10 ml single-use syringes (B. Braun Melsungen AG H4606108V)
- Disposable sterile syringe filters, 25 mm, 0.20 Micron, cellulose acetate membrane acrylic (IWAKI 2052 – 025)

5.13.3 Reagents

- Ammoniumacetate (Merck 1116)
- NaCl (Merck 1.06404.1000)
- KCl (Merck 4936.1000)
- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (Merck 1.06580.1000)
- Glucose (Merck 4074.0500)
- Hepes, free acid (Sigma 3375)
- Ethanol (Merck 1.00983 – 1000)
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck 1.02382.1000)
- Glycerol (cell culture tested, Sigma G-2025)
- Geneticin (G 418 solution disulfate salt; Sigma G-7034)

5.13.4 Buffers

PBS (see 5.1.4)

Medium for BeWo cells (see 5.1.4)

7.5 M Ammoniumacetate

- Dissolve 28.91 g Ammoniumacetate in a final volume of 50 ml A.bid
- Store at rt

Hepes buffer, pH 7.1

- 0.8 g NaCl (137 mM)
- 0.037 g KCl (5 mM)
- 0.012 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (0.7 mM)
- 0.108 g glucose (6 mM)
- 0.500 g Hepes (21 mM)
- Dissolve in 90 ml A.bid.
- adjust to pH 7.1

- Bring to a final volume of 100 ml A.bid.
- filter to sterilize
- Store in aliquots (e.g. 5 ml) at -20°C

2 M CaCl₂

- Dissolve 14.7 g CaCl₂·2H₂O in a final volume of 50 ml A.bid.
- filter to sterilize
- stored at rt

15% glycerol in PBS

- Add 15 ml glycerol (15 % v / v) to 85 ml PBS
- filter to sterilize
- store at rt

5.13.5 Transfection protocol [402]

- Grow cells in 75 cm² flasks to 50 – 70% confluency - cells should be in a good condition! Prepare 1 flask/1DNA sample

Day 1

- Prepare samples - precipitate plasmid of interest (HA-tagged MT1 in pcDNA3) in microfuge tube
 - o Use 50 µl of plasmid DNA (0.72 µg / µl)
 - o Add 190 µl A.bid.
 - o Add 100 µl 7.5 M ammoniumacetate
 - o Add 600 µl ethanol
- Incubate for 10 min at -80°C
- Spin for 10 min (14.000 x g) in microcentrifuge (gives a pellet of DNA)
- Transfer tube to laminar flow and work sterile from now on
- remove supernatant by aspiration
- Dissolve pellet completely in 500 µl Hepes buffer, pH 7.1 (pH is important)
- Prepare 1 tube that contains no DNA
- Add 31 µl 2 M CaCl₂, and mix
- incubate for 45 min at rt
- Meanwhile detach cells by e.g. trypsinization, collect cells from 75 cm² flasks in 15 ml medium. Collect cells by centrifugation for 5 min at 200 x g, remove supernatant
- Transfer DNA-precipitate-solution (or negative control) to cell pellet, mix carefully and incubate for another 20 min at rt
- Add 15 ml complete medium to each tube and plate the cells in 10 cm dishes

- Incubate in CO₂ incubator on

Day 2

- Check cells - small black aggregates of Calcium phosphate are a good sign!
- Perform a glycerol shock to enforce DNA uptake:
- Replace medium of cells with 5 ml 15 % glycerol in PBS
- Leave on cells for 2 min
- Remove and wash with 15 ml complete medium
- Incubate with complete medium on

Day 3

- Split cells 1:3 (MR1) and start the selection of transfected cells by adding the required antibiotic to the medium (the pcDNA3 encodes a geneticin resistance)
- For G418 (Geneticin) the concentration for BeWo cells is 0.25 mg / ml complete medium

Day X

This is usually 2 – 3 weeks later, when most of the cells have died, but some colonies have formed and are large enough to be picked (=transferred to a new dish)

- Prepare sterile cloning rings (e.g. cut the upper part of blue tips with a razor blade thereby obtaining rings of 1 cm high. Sterilize them in ethanol.
- Autoclave grease in a glass Petridish
- Wash cells with PBS, remove PBS
- Dip rings with 1 side in grease and 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 and wait until cells detach
- Transfer cells of 1 clon into 1 well of a 24 well plate, add 1 ml of medium
- Expand cells and when there are enough cells test ~12 clons by (usually) immunofluorescence for expression of transfected protein

5.14 RNA Isolation**5.14.1 Background**

RNA can be isolated in a single-step method termed guanidinium thiocyanate-phenol-chloroform extraction method [76]. Guanidinium thiocyanate (GTC) is a chaotropic agent that lyses cells and inactivates RNases and other enzymes at the same time.

RNA is separated from DNA after extraction with an acidic solution containing GTC, sodium acetate, phenol and chloroform, followed by centrifugation under acidic conditions. Total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in

the interphase or in the lower organic phase. Total RNA can be recovered by precipitation with isopropanol and used for RT-PCR experiments.

5.14.2 Materials

- Microcentrifuge (Eppendorf centrifuge 5415D)
- Thermomixer comfort (Eppendorf AG)
- U-2000 spectrophotometer (Hitachi)
- Mortar and pestle (stored at -80°C)
- DNase, RNase, DNA free tubes 1.5 ml (Biozyme, 1.6 ml reaction tubes, DNase, RNase free, 710310)
- DNase, RNase, DNA free Pipette tips
 - o 1250 µL (e.g. Biozym, Safe seal tips® professional, Filter Tip, 770600)
 - o 200 µL (e.g. Biozym, Safe seal tips® professional, Filter Tip „XL“ low binding, 770280)
- Cell scrapers, sterile (Iwaki 9010 – 320)
- Latex Gloves (e.g. Hartmann, Peha-Soft® powderfree, 942162)

5.14.3 Reagents

- peqGOLD TriFast™ extraction system for RNA (Pqrlab 30 – 20XX)
- Chloroform (Merck 2445)
- Isopropanol (Merck 1.09634.1011)
- Ethanol, 75 % (diluted from Ethanol absolute, VWR Prolabo, 20821.310)
- DNase, RNase free distilled water, “PCR water” (Gibco®, Invitrogen™, Ultrapure™, 0977035)

5.14.4 RNA isolation from cells

Attention: Phenol and GTC are harmful. Use gloves, protection goggles and work in a fume hood. In addition, wear gloves and use RNase free solutions and materials to prevent RNase contamination.

- Cells (25 cm²), 70 – 100 % confluency
- Remove medium and wash cells with 10 ml PBS
- Add 1 ml PBS and harvest cells with a cell scraper
- Centrifuge cell suspension at 200 x g for 5 min at 4 °C
- Remove sup
- All following RNA extraction steps are carried out on ice or at 4 °C in the coldroom
- Add 1 ml TriFast™ Reagent and lyse pellet by repetitive pipetting
- Incubate lysate for 5 min at rt

- Add chloroform (200 μ l / 1 ml TriFast™ Reagent)
- Shake tube vigorously for 15 sec, then incubate for 5 min at rt
- Centrifuge for 15 min at 12.000 x g
- Remove the upper aqueous phase carefully and transfer to fresh tube containing 500 μ l isopropanol
- Incubate samples for 10 min at rt
- Centrifuge for 10 min at 12.000 g and remove (isopropanol) sup
- Wash pellet with 1 ml EtOH (75 %)
- Centrifuge for 5 min at 12.000 x g, and remove (EtOH) sup
- Dry pellets in a thermomixer at 55 °C (for max. 3 min), do not dry completely, otherwise RNA becomes insoluble
- Add 20 μ l DEPC treated water and incubate lysate for 10 min at 55 °C to dissolve RNA, centrifuge shortly
- RNA is stored at -80 °C

5.14.5 RNA quantitation and purity determination

The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 40 μ g of RNA per ml. This is valid for measurements in water. A ratio of readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that adsorb in the UV, such as proteins.

- To determine the RNA content, mix 398 μ l DEPC treated water with 2 μ l of the RNA solution
- After vortexing the samples, measure the absorption in a spectrophotometer at 260 nm and 280 nm (re-zero the spectrophotometer using water)
- Multiplicating the A_{260} value with the factor of 8 results in the amount of μ g RNA in 1 μ l water (μ g / μ l) ($[RNA]$ = RNA concentration, dilutionfactor = 200)

$$[RNA] = \frac{40 * A_{260} * dilutionfactor}{1000} = \mu g / \mu l$$

- The A_{260}/A_{280} ratio can be used as an indicator for RNA purity and it should be >1.6
- To control for possible RNA degradation, the newly extracted RNA is routinely analyzed by native agarose gel electrophoresis (see 5.8)

5.15 Reverse Transcription

5.15.1 Background

In Reverse Transcription (RT reaction or first strand cDNA synthesis) single-stranded RNA (total cellular RNA or poly (A) RNA) is reverse transcribed into complementary DNA (cDNA) by using a reverse transcriptase enzyme, a primer (e.g. random hexamer primer), desoxynucleosidetriphosphate (dNTPs) and an RNase inhibitor. The resulting cDNA can be used in RT-PCR reactions.

5.15.2 Materials

- Personal Cyclor (Biometra)
- PCR tubes (Soft strips and Cap strips, DNA, DNase, RNase and PCR-Inhibitor-free, Biozyme 711630 and 711640)
- Safe-Seal Tips (PeqLab, e.g. 20 µl #81–1020, 200 µl #81–1040, 1000 µl #81–1050)

5.15.3 Reagents

- High capacity cDNA Reverse Transcription kit (Applied Biosystems 4368814) containing:
 - o RNase free water (PCR water)
 - o Ribonuclease inhibitor
 - o Reverse transcriptase
 - o Random hexamer primer
 - o 10mM desoxynucleosidetriphosphate (dNTP) mix
 - o 5X reaction buffer

5.15.4 Reverse transcription

- Adjust all RNA samples to a RNA concentration of 0.5 µg / µl
- Take 2 µg total RNA
- Add PCR water to a total volume of 10 µl
- Prepare a reverse transcription master mix

10 x buffer	2 µl
25 x dNTPs (100mM)	0.8 µl
10 x RT Random primer	2 µl
MultiScribe Reverse Transcriptase	2 µl
RNase inhibitor	1 µl
PCR water	3.2 µl
- Centrifuge the samples briefly
- Add 10 µl mastermix to each sample

- Incubate the samples in the personal cyclor
 - 10 min at rt (Primer annealing to RNA)
 - 120 min at 37 °C (RT reaction)
 - 5 min at 85 °C (inactivation of enzyme)
 - ∞ 4 °C
- These conditions are optimized for the use with the High Capacity cDNA Reverse Transcription Kit!!!
- The resulting cDNA can be stored at -20 °C

5.16 RT-PCR for MT1 and β -actin amplification

5.16.1 Materials

- Personal Cyclor (Biometra)
- PCR tubes (Soft strips and Cap strips, DNA, DNase, RNase and PCR-Inhibitor-free, Biozyme 711630 and 711640)
- Safe-Seal Tips (PeqLab, e.g. 20 μ l #81–1020, 200 μ l #81–1040, 1000 μ l #81–1050)

5.16.2 Reagents

- cDNA, stored at -20 °C
- Ultrapure DNase/RNase free water (Invitrogen 10977035)
- Taq DNA Polymerase (1 U / μ l; Fermentas EP0404), stored at -20 °C
- 10x Taq DNA polymerase (Fermentas EP0404), stored at -20 °C
- 25 mM $MgCl_2$ (Fermentas EP 0404), stored at -20 °C
- 10 mM (each) dNTP mix (Fermentas EP 0404), stored at -20 °C
- Loading dye solution (Fermentas R 0611)
- Marker: Fermentas Gene Ruler 100 bp marker (Fermentas SM0241).
- Sense and antisense primers (1 μ m each), stored at -20 °C

MT1 sense primer [44]: 5'-TCCTGGTCATCCTGTCGGTGTATC-3'

MT1 antisense primer [44]: 5'-CTGCTGTACAGTTTGTCGTAAGT-3'

β -actin sense primer [417]: 5'-ATCTGGCACCACCTTCTACAATGAGCTGCG-3'

β -actin antisense primer [417]: 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'

5.16.3 Protocol for PCR reaction

- Prepare the PCR Mastermix (12.5 μ l) for MT1

10x Taq DNA Polymerase	2.5 μ l
$MgCl_2$ (25 mM)	1.0 μ l
dNTP mix (10 mM each)	0.5 μ l

- | | |
|--------------------------------|--------------|
| Primer sense (1 μ M) | 0.25 μ l |
| Primer antisense (1 μ M) | 0.25 μ l |
| Water | 7.5 μ l |
| Taq Polymerase (1 U / μ l) | 0.5 μ l |
- Prepare the PCR Mastermix (12.5 μ l) for β -actin

10x Taq DNA Polymerase	2.5 μ l
MgCl ₂ (25 mM)	1.5 μ l
dNTP mix (10 mM each)	1 μ l
Primer sense (1 μ M)	0.25 μ l
Primer antisense (1 μ M)	0.25 μ l
Water	6 μ l
Taq Polymerase (1 U / μ l)	1 μ l
 - Calculate the volumes according to the number of samples (n) being tested and add 5 % excess volume
*Formula: $[(n + 1) * 1.05] * (\text{vol for single reaction}) = \text{needed volume } [\mu\text{l}]$ e.g the amount of 10x buffer for 10 samples would be 28.9 μ l*
 - Distribute 12.5 μ l of PCR mastermix for each sample to a 0.2 ml PCR strip tube
 - Add 12.5 μ l of diluted template DNA.
 - Run PCR
 - PCR cycling program for MT1:

1. 94 $^{\circ}$ C	8 min
2. 94 $^{\circ}$ C	30 s
3. 56 $^{\circ}$ C	45 s
4. 72 $^{\circ}$ C	1 min
5. 68 $^{\circ}$ C	7 min
6. 4 $^{\circ}$ C	∞
- Repeat steps 2 – 4 x 40 times
- PCR cycling program for β -actin:

1. 94 $^{\circ}$ C	2 min
2. 94 $^{\circ}$ C	30s
3. 61 $^{\circ}$ C	1 s
4. 72 $^{\circ}$ C	1 min
5. 72 $^{\circ}$ C	5 min
6. 4 $^{\circ}$ C	∞
 - Repeat steps 2 – 4 x 25 times
 - After the PCR run, analyse fragments by native agarose gel electrophoresis (see 5.11)

5.17 TaqMan Quantitative reverse transcriptase PCR

5.17.1 Materials

- Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA)
- MicroAmp Fast 96 - well reaction plate (Applied Biosystems 4346907)
- MicroAmp Optical Adhesive Film (Applied Biosystems 4311971)
- Safe-Seal Tips (PeqLab, e.g. 20 µl #81 – 1020, 200 µl #81 – 1040, 1000 µl #81 – 1050)

5.17.2 Reagents

- Mastermix (TaqMan Gene Expression MM, AB 4369514)
- MTNR1A assay (AB Hs00195567_m1)
- HPRT assay (AB 4310890E)

5.17.3 Protocol

- Prepare the PCR Mastermix (5.5 µl / sample)

Mastermix	5 µl
MTNR1A or HPRT assay	0.5 µl
Water	0.5 µl
- Calculate the volumes according to the number of samples (n) being tested and add 5 % excess volume
*Formula: $[(n + 1) * 1.05] * (\text{vol for single reaction}) = \text{needed volume } [\mu\text{l}]$ e.g the amount of 10x buffer for 10 samples would be 28.9 µl*
- Distribute 5.5 µl of PCR mastermix for each sample to a well of the 96 well reaction plate
- Add 4 µl (10 ng) of diluted template DNA (use duplicates).
- Seal plate with adhesive film
- Run PCR to determine relative levels of expression of MT1
- Relative mRNA expression levels of MT1 were calculated by the DDCT (threshold cycle) method normalized to the expression of the housekeeping gene HPRT [403]
- All calculations were done according to the manufacturer's instructions

6 RESULTS

6.1 Endogenous expression and localization of melatonin receptor 1 (MT1) protein in BeWo cells

The binding regions of three different primary anti-MT1 antibodies used in this study are depicted in Figure 6.1. sc-13179 should recognize the N-terminus of human MT1 and has been used to demonstrate MT1 expression in human cell lines and tissue by western blotting and immunofluorescence (e.g. [365, 44, 404]). The epitopes of Ab-13035 and Ab -13036 are localized in the 2nd and 3rd intracellular loop of human MT1, respectively. To our knowledge, only Ab-13036 has so far been applied in immunofluorescence microscopic studies [405].

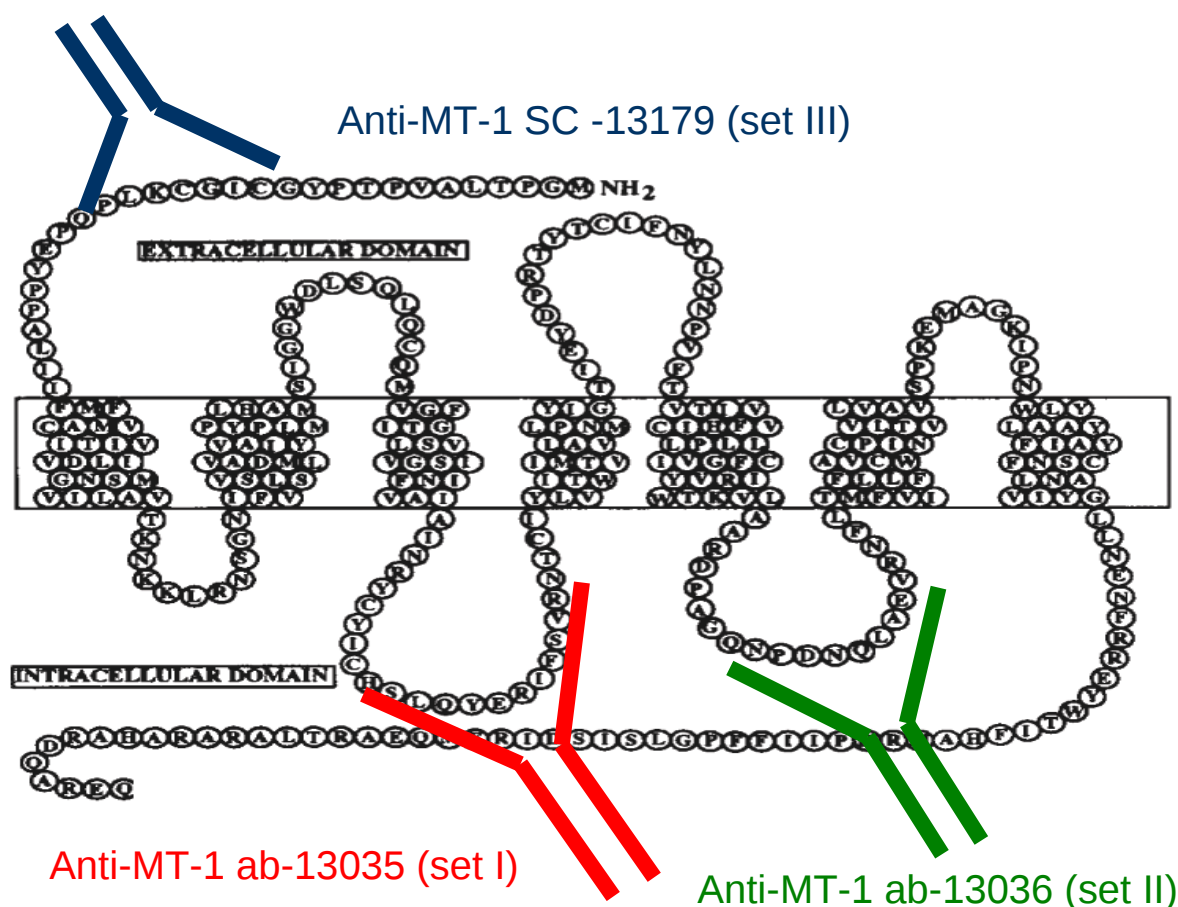


Figure 6.1 Schematic depictions of the binding sites of three anti-MT1 antibodies to MT1 (modified from 398)

To study endogenous expression of MT1 protein in the BeWo cell line and in the human term placenta, cell and tissue homogenates were prepared according to (see 5.2) and the protein concentration was determined as described in (see 5.3). Same amounts of protein were separated by SDS-PAGE and transferred to PVDF-membranes as detailed in (see 5.4). MT1 was analysed using different sets of antibodies combining MT1-specific primary and HRP-labeled secondary antibodies (Set I-III; see table 5.1) and detection of HRP-labeled proteins was performed as described in (see 5.4.6).

To analyse endogenous localisation of MT1 in BeWo cells, immunofluorescence microscopy was applied (see 5.5). Cells were seeded on coverslips (see 5.5.5) and, two days later, were stained according to the protocol detailed in (see 5.5.6) for MT1 using different sets of antibodies combining MT1-specific primary and Alexa-Fluor-568-conjugated secondary antibodies (Set I-III; see table 5.4.). For analysis of MT1 expression by Ab-13035 in placental tissue, placentas were collected as indicated in (see 5.1.5), processed for cryosectioning (see 5.5.7 and 5.5.8) and an immunofluorescence staining was performed on the cryosections as detailed in (see 5.5.9). Finally, cells and tissue samples were analyzed by immunofluorescence confocal laser microscopy.

6.1.1 Western blot analysis with anti-MT1 antibody Ab-13035

First, a western blot analysis with anti-MT1 antibody Ab-13035 (antibody set I, Table 5.1) was performed on term placental lysates as well as BeWo cell lysates (20 µg / lane). The results are depicted in (Figure 6.2a). In control incubations, the primary antibody was omitted (Figure 6.2b).

Western blot analysis using anti-MT1 antibody Ab-13035 revealed one major band at approximately 90 kDa in all samples investigated. Upon prolonged exposure, additional bands appeared (Figure 6.2.c). One of these bands had a molecular weight of 130 kDa, while several bands became visible in the range between 40 and 90 kDa. Cell lysates of the human osteosarcoma cell lines MG63 and HOS that were used as additional positive controls based on the previously published expression of MT1 mRNA in these cells [389], exhibited an identical staining pattern (data not shown).

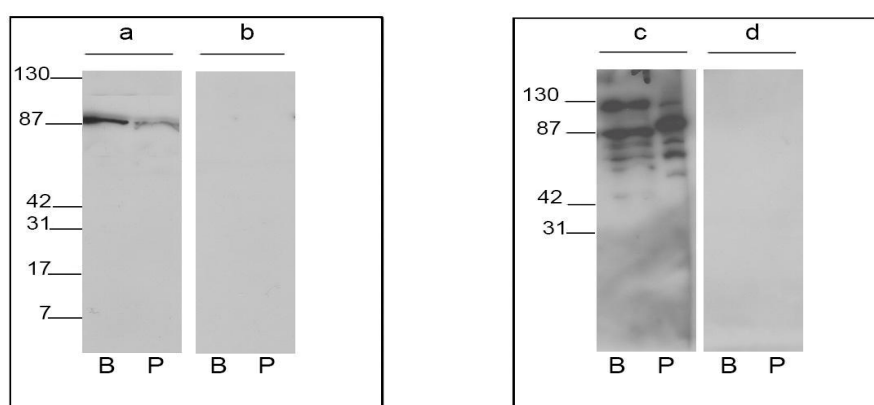


Figure 6.2 Western blot analysis of MT1 in (B) BeWo cells and (P) human term placenta.

Similar amounts of protein (20 µg) were separated by SDS-PAGE and transferred to PVDF membranes. Two identical membranes (a/c+b/d) were prepared. In (a/c), the membrane was incubated with “antibody set I” containing the primary antibody “anti-MT1 (Ab-13035)”, while in (b/d) the primary antibody was omitted. In (c+d), exposure times were prolonged. Numbers indicate the position for each protein marker in kilodalton.

6.1.2 Immunofluorescence analysis

Next, usability of the anti-MT1 antibody Ab-13035 in microscopic studies was tested. Formaldehyde-fixed and permeabilized BeWo cells were incubated with anti-MT1 antibody Ab-13035 in combination with an Alexa-568-linked secondary antibody (Antibody set I, Table 5.4). Cells were analyzed by confocal laser microscopy, all pictures were processed identically and representative optical sections from top and bottom layers of cells are shown in (Figure 6.3 a+b), respectively. Identically treated cells were used in control incubations, where the primary antibody was omitted (Figure 6.3 c+d). Ab-13035 staining of BeWo cells resulted in a vesicular staining pattern with the majority of staining localized to intracellular compartments.

In addition to BeWo cells, formaldehyde-fixed cryosections of human term placenta were incubated with the anti-MT1 antibody Ab-13035 in combination with an Alexa-568-linked secondary antibody (Antibody set I, Table 5.4). Identically treated tissue was used in control incubations, where the primary antibody was omitted (data not shown). Nuclei in placental sections were stained with DRAQ5 (blue). Samples were analyzed by confocal laser microscopy and two representative optical sections are shown in Figure 6.3 e and f. Autofluorescence of tissue at Ex488/Em520 is included for better visualization of tissue. The protein (MT1?) detected by Ab-13035 and shown in red was mainly expressed by the STB covering the chorionic villi as indicated by the arrowheads in (Figure 6.3 e and f), localizing predominantly to intracellular compartments.

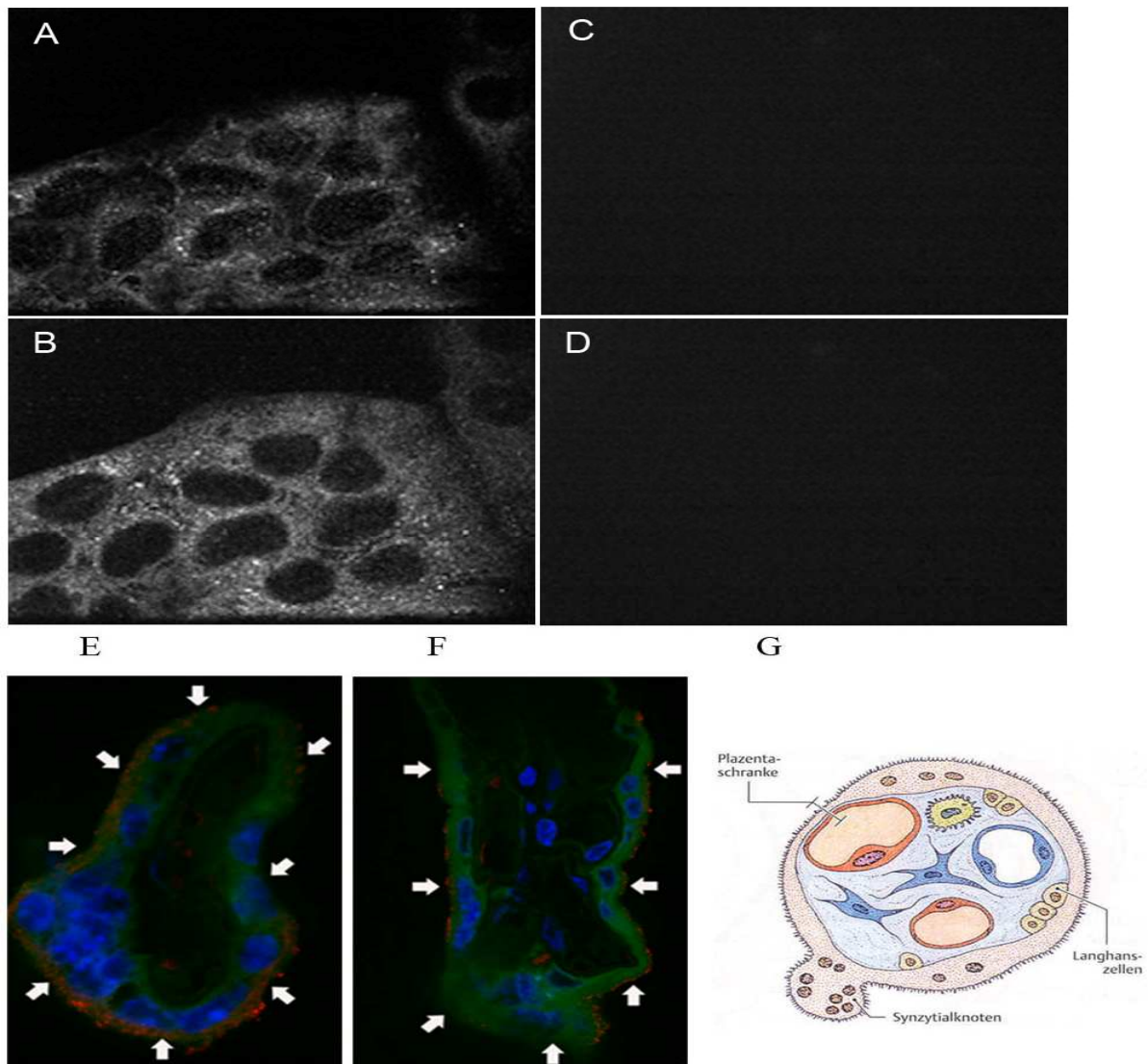


Figure 6.3 Localization of MT1 in BeWo cells and in third trimester human placental chorionic villi.

(a+b) Formaldehyde-fixed and permeabilized cells were immunostained for expression of MT1 using the primary antibody anti-MT1 Ab-13035 and the respective secondary Alexa-Fluor 568-conjugated antibodies. (c+d) Respective control incubations, where the primary antibodies were omitted, but cells were otherwise processed under identical conditions. Cells were analyzed by confocal laser microscopy. Two optical sections from the top (a+c) or bottom (b+d) region of the cells are shown. MT1 expression was detected and primarily localized to intracellular compartments.

(e+f) Formaldehyde-fixed placental cryosections were immunostained for expression of MT1 using the primary antibody anti-MT1 (Ab-13035) and a secondary Alexa-568-conjugated antibody. Nuclei were stained with DRAQ5 (blue) and sections were analyzed by confocal laser microscopy. Representative optical sections of two chorionic villi are shown. MT1 localization (red) is found mainly in the syncytiotrophoblast layer (STB, arrowheads) when compared to (g). Tissue autofluorescence is detected at Ex 488/Em 520 and is shown in green. Control incubations, where the primary antibody was omitted were analyzed with the same microscope settings and stained negative (data not shown). (g) Schematic depiction of a term placental chorionic villus.

6.1.3 Western blot analysis with anti-MT1 antibody Ab-13036

Next, a western blot analysis with anti-MT1 antibody Ab-13036 (antibody set II, Table 5.1) was performed on term placental lysates as well as BeWo cell lysates (20 µg / lane). The results are depicted in (Figure 6.4a). In a control incubation, the primary antibody was omitted (Figure 6.4b).

Western blot analysis using anti-MT1 antibody Ab-13036 revealed 4 major bands in all of the samples investigated. A pair of bands was observed at 40 kDa and above (50-60 kDa), while a second pair of bands appeared at 90 and approximately 130 kDa. Again, when MG63 and HOS cell lysates were used as additional positive controls for MT1 expression they exhibited an identical staining pattern (data not shown).

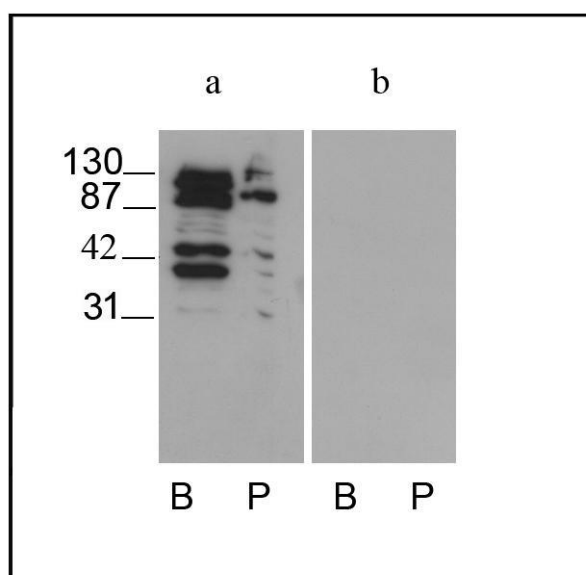


Figure 6.4 Western blot analysis of MT1 in (B) BeWo cells and (P) human term placenta.

Similar amounts of protein (20 µg) were separated by SDS-PAGE and transferred to PVDF membranes. Two identical membranes (a+b) were prepared. In (a), the membrane was incubated with “antibody set I” containing the primary antibody “anti-MT1 (Ab-13036)”, while in (b) the primary antibody was omitted. Numbers indicate the position for each protein marker in kilodalton.

6.1.4 Immunofluorescence analysis

Next, usability of the anti-MT1 antibody Ab-13036 in microscopic studies was investigated. Formaldehyde-fixed and permeabilized BeWo cells were incubated with this primary antibody in combination with an Alexa-568-linked secondary antibody (Antibody set II, Table 5.4). Cells were analyzed by confocal laser microscopy and representative optical sections from top and bottom layers of cells are shown in (Figure 6.5 a, b, e and f). Identically treated cells were used in control incubations, where the primary antibody was omitted (Figure 6.5 c, d, g and h).

As observed for A-13035, BeWo cells stained brightly and the majority of the protein (MT1?) stained with Ab-13036 localized to intracellular compartments (Figure 6.5 a and b).

However, on some occasions additional staining of the nuclear membrane was observed (Figure 6.5 e and f).

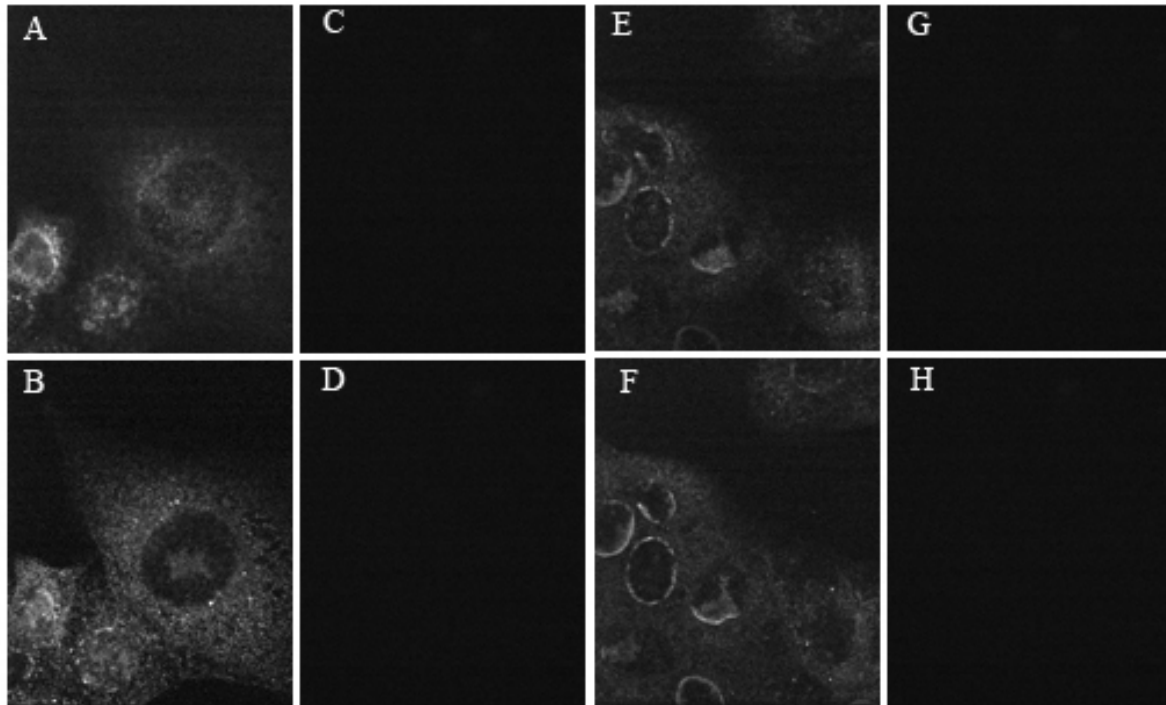


Figure 6.5 Localization of MT1 in BeWo cells.

(a+b, e+f) Formaldehyde-fixed and permeabilized cells were immunostained for expression of MT1 using the primary antibody anti-MT1 Ab-13036 and the respective secondary Alexa-Fluor 568-conjugated antibodies. (c+d, g+h) Respective control incubations, where the primary antibodies were omitted, but cells were otherwise processed under identical conditions. Cells were analyzed by confocal laser microscopy. Two optical sections from the top (a+c, e+g) or bottom (b+d, f+h) region of the cells are shown. MT1 expression was detected and primarily localized to intracellular compartments (a,b). On some occasions, additional nuclear membrane staining was observed (arrows in e,f).

6.1.5 Western blot analysis with anti-MT1 antibody sc-13179

Finally, the anti-MT1 antibody sc-13179 (antibody set III, Table 5.1) was tested in western blot analysis of term placental lysates as well as BeWo cell lysates (40 µg / lane) (Figure 6.6a). In a control incubation, the primary antibody was omitted (Figure 6.6b).

Western blot analysis using anti-MT1 antibody sc-13179 revealed 1 major band with a molecular weight of 40 kDa in all of the samples investigated.

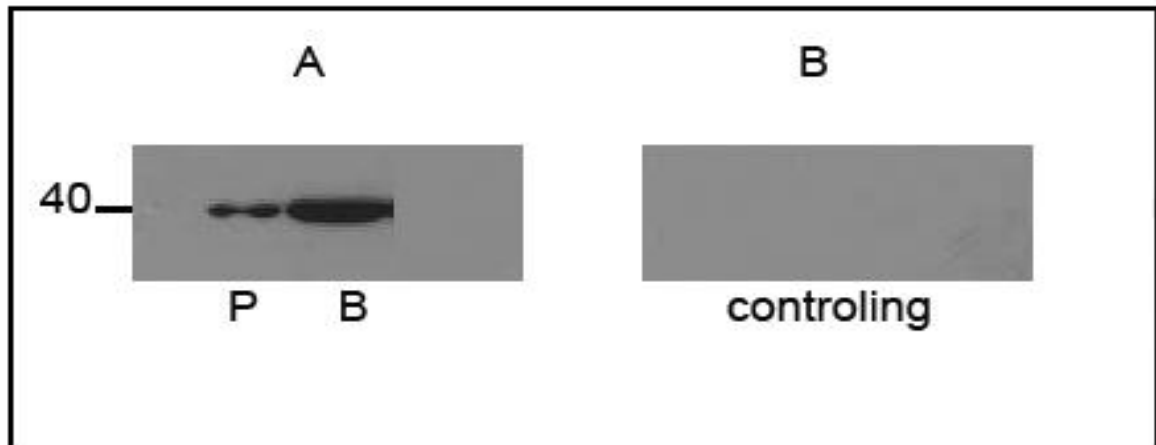


Figure 6.6 Western blot analysis of MT1 in (P) Placenta and (B) BeWo cells.

Similar amounts of protein (40 μ g) were separated by SDS-PAGE and transferred to PVDF membranes. Two identical membranes (a+b) were prepared. In (a), the membrane was incubated with “antibody set I” containing the primary antibody “anti-MT1 (sc-13179)”, while in (b) the primary antibody was omitted. Numbers indicate the position for each protein marker in kilodalton.

The experiment was repeated under identical conditions but including cell lysate from the ovarian cancer cell line OVCAR-3. MT1 expression in OVCAR-3 cells has been demonstrated [390]. Again 1 major band with a molecular weight of 40 kDa was detected in all samples investigated (Figure 6.7a). When the blot was exposed overnight, faint additional bands of higher molecular weight (between 40 and 90 kDa) became visible.

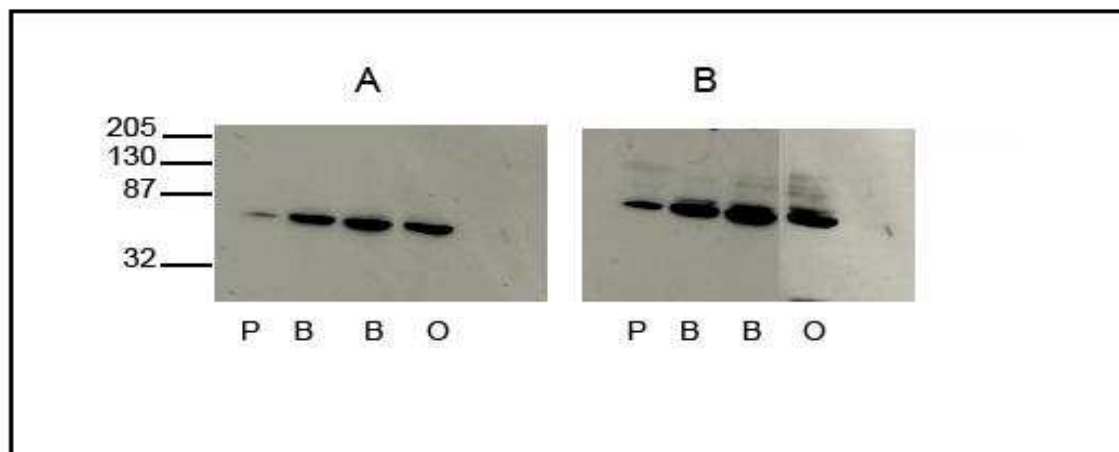


Figure 6.7 Western blot analysis of MT1 in (P) Placenta, (B) BeWo cells (2 different preparations of cell lysates), and (O) OVCAR-3 cells.

Similar amounts of protein (40 μ g) were separated by SDS-PAGE and transferred to PVDF membranes. In (a+b), the membrane was incubated with “antibody set I” containing the primary antibody “anti-MT1 (sc -13179)”; in (a) the membrane was exposed for 1 h, while in (b) the membrane was exposed overnight. Numbers indicate the position for each protein marker in kilodalton.

The specificity of the anti-MT1 antibody sc-13179 was next investigated using a commercially available blocking peptide (sc-13179 P). BeWo cell lysates as well as HOS and MG63 [389] lysates (40 µg / lane) were analysed by western blotting to detect MT1 expression and two identical PVDF membranes were prepared (Figure 6.8a and b). Before incubation with the PVDF membrane, the anti-MT1 antibody sc-13179 was either preincubated for 2 h with the blocking peptide (Figure 6.8.b) or with PBS (Figure 6.8.a) as detailed in (see 0). Thereafter, the blots were processed identically. As shown in Figure 6.8a, western blot analysis using anti-MT1 antibody sc-13179 preincubated with PBS again revealed 1 major band with a molecular weight of 40 kDa in all of the samples investigated (Figure 6.8a). In contrast, no band was detected upon preincubation of the antibody with the blocking peptide (Figure 6.8b), indicating specificity of the antibody for a part of the MT1 sequence.

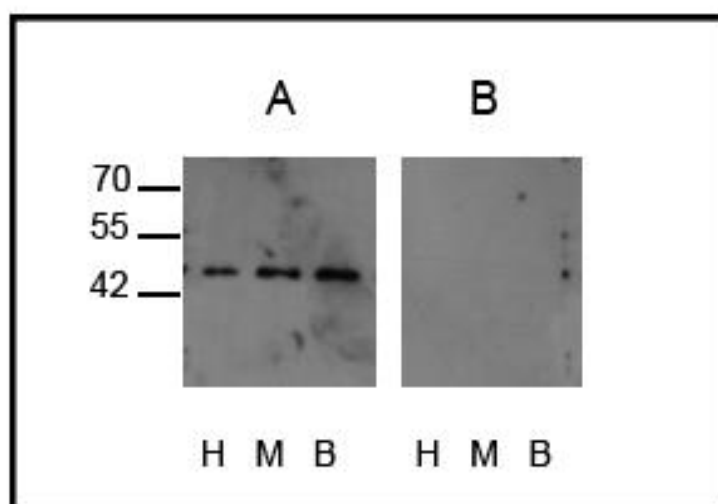


Figure 6.8 Western blot analysis of MT1 in (H) HOS, (M) MG63 and (B) BeWo cells.

Similar amounts of protein (40 µg) were separated by SDS-PAGE and transferred to PVDF membranes. In (a), the membrane was incubated with “antibody set I” containing the primary antibody “anti-MT1 (sc-13179)” preincubated with PBS, while in (b) the membrane was incubated with “antibody set I” containing the primary antibody “anti-MT1 (sc-13179)” preincubated with a corresponding blocking peptide. Preincubation with the blocking peptide significantly reduced the intensity of the band detected. Numbers indicate the position for each protein marker in kilodalton.

6.1.6 Immunofluorescence analysis

Finally, formaldehyde-fixed and permeabilized BeWo cells were incubated with anti-MT1 sc-13179 primary antibodies in combination with an Alexa-Fluor 568-linked secondary antibody (Antibody set III, Table 5.4). Cells were analyzed by confocal laser microscopy and representative optical sections from top and bottom layers of cells are shown in (Figure 6.9 a

and b). Identically treated cells were used in control incubations, where the primary antibody was omitted (Figure 6.9 c and d).

Again, BeWo cells exhibited a bright and mainly punctuated cellular staining indicating that the majority of the protein (MT1?) detected by sc-13179 localized to intracellular compartments.

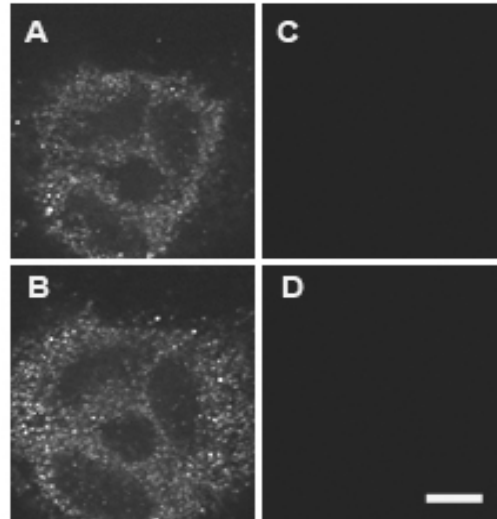


Figure 6.9 Localization of MT1 in BeWo cells.

(a+b) Formaldehyde-fixed and permeabilized cells were immunostained for expression of MT1 using the primary antibody anti-MT1 sc-13179 and the respective secondary Alexa-Fluor 568-conjugated antibodies. (c+d) Respective control incubations, where the primary antibodies were omitted, but cells were otherwise processed under identical conditions. Cells were analyzed by confocal laser microscopy. Two optical sections from the top (a+c) or bottom (b+d) region of the cells are shown. MT1 expression was detected and primarily localized to intracellular compartments. Bar represents 10 μ m.

In summary, three different commercially available anti-MT1 antibodies were tested for their ability to detect endogenous MT1 protein in BeWo choriocarcinoma cells, human placental tissue and other cell lines (HOS, MG63, OVCAR-3) recently demonstrated to express at least MT1 mRNA [365, 44, 389, 390] by western blotting and immunofluorescence microscopy.

In western blotting as well as immunofluorescence microscopy, all antibodies tested reacted positively and identical staining patterns were observed in all cell lines with demonstrated expression of MT1 mRNA. However, while the antibodies Ab-13035 and Ab-13036 directed against intracellular domains of the receptor detected several bands in the range of 40 – 130 kDa, anti-MT1 antibody from sc (sc-13179) reproducibly detected only one band of approximately 40 kDa.

In immunofluorescence microscopy, the pattern of localization of the proteins detected by these antibodies was very similar: localization was observed primarily in vesicular,

intracellular compartments, while plasma membrane staining was not observed in BeWo cells. With Ab-13036 some additional staining of the nuclear membrane was observed.

Soon after these initial studies to characterize the antibodies, Ab-13035 and Ab-13036 were no longer available from their company. Therefore, only sc-13179 recognizing a 40 kDa protein with a mainly intracellular localisation was further characterized.

6.2 Stable transfection of BeWo cells with human melatonin receptor 1

To confirm and further characterize the identity of the 40 kDa protein detected by western blotting with the anti-MT1 antibody sc-13179 in lysates of BeWo cells (and human placenta), we decided to generate a BeWo cell line overexpressing the human MT1.

6.2.1 Amplification, purification and analysis of the plasmid

The plasmid pcDNA3 (Invitrogen) containing the coding region of the human melatonin receptor 1, MT1, cDNA was a generous gift of Dr. Tarja Kokkola (Inst. of Genetics, Univ. Medical Center Hamburg-Eppendorf, Hamburg Germany). To improve detection, a triple hemagglutinin (HA) epitope tag had been subcloned after the initiating Met codon of the WT receptor (Figure 6.10). Triple HA tag contains three nine-amino acid sequences (YPYDVPDYA) derived from the influenza virus HA protein and has higher affinity to antibodies than a single HA tag [397]. The plasmid pcDNA3 contains resistances to ampicillin and neomycin for selection in bacteria and mammalian cells, respectively.

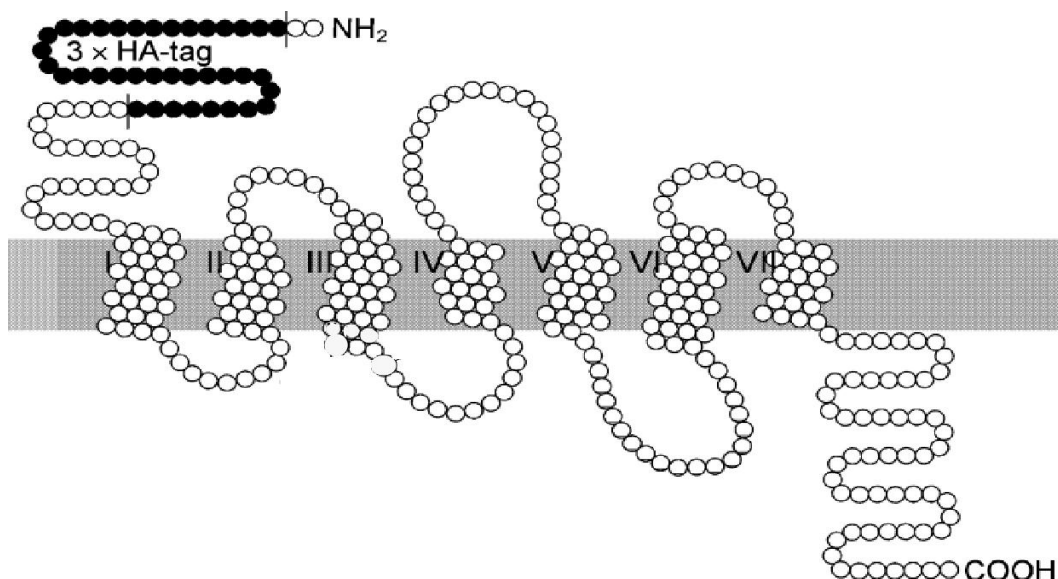


Figure 6.10. Schematic depiction of the human MT1 melatonin receptor containing the N-terminal triple HA epitope tag (modified from 398)

In order to amplify the plasmid (mel1a-3xHA-pcDNA3), 50 μ l of competent bacteria (prepared as described in see 5.6.5) were transformed with 20 μ g as well as 40 μ g of the plasmid DNA as detailed in (see 5.6.6). From each transformation, 20 μ l as well as 200 μ l transformed bacteria were spread on ampicillin-containing agar plates and incubated over night at 37 $^{\circ}$ C.

The next day, 12 clones were picked from plates with low clone density (i.e. 20 μ g plasmid DNA, 20 μ l transformed bacteria) and expanded in 2 ml LB-medium containing ampicillin as described in (see 5.7.4). Aliquots (1.5 ml) of these cultures were used for plasmid DNA purification applying an alkaline lysis protocol (see 5.7.4). DNA purified from each of these minipreps was dissolved in 50 μ l A.bid. and used to confirm the identity of the plasmid.

As an initial control, restriction enzyme digestion was performed on all 12 DNA samples as outlined in (see 5.9). Restriction enzyme digestion of mel1a-3xHA-pcDNA3 using the enzyme HindIII should result in 2 DNA fragments of 5.5. kb and 1.1. kb, respectively, representing the vector and its insert. Digestion products were mixed with loading buffer and separated on 1 % agarose gels as described in (see 5.11). As shown in (Figure 6.11), 2 bands of appropriate size were detected in all samples indicating the correct identity of the plasmid.

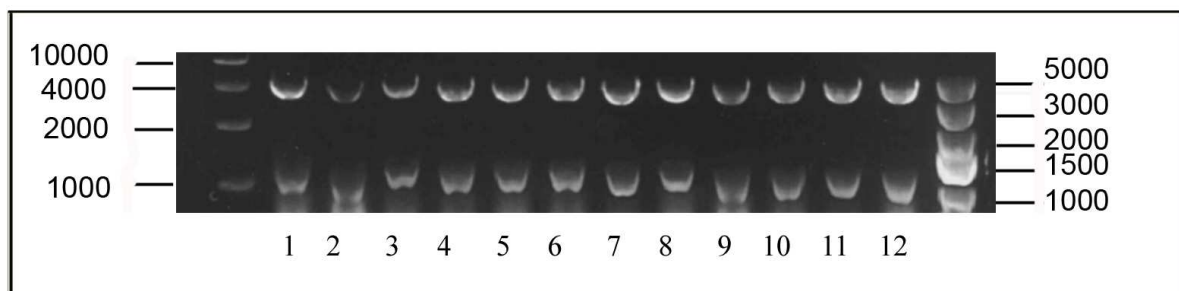


Figure 6.11. Analysis of mel1a-3xHA-pcDNA3 by restriction enzyme digestion using HindIII.

The presence of two DNA fragments of 1.1kb (mel1a-3xHA insert) and 5.5 kb(vector) size, respectively, following Hind III restriction enzyme digestion was verified in 12 plasmid minipreps.

Next, PCR was used to partially verify the MT1 sequence. Primers were selected according to [400] and thus should result in a PCR product of 286 bp size. The localization of the primer sequences within the sequence of MT1 is shown in (Figure 6.12).

```

5' -
GGGACGCGAACAGGGACCATGCAGGGCAACGGCAGCGCTGCCAACGCCTCCCAGCCC
GTGCTCCGCGGGGACGGCGCGGGCCCTCGTGGCTGGCGTCCGCCCTGGCCTGCGTCCTC
ATCTTCACCATCGTGGTGGACATCCTGGGCAACCTCTGGTCATCCTGTCGGTGTATCGG
AACAAGAAGCTCAGGAACGCAGGAAACATCTTTGTGGTGAGCTTAGCGGTGGCAGACCTG
GTGGTGGCCATTTATCCGTACCCGTTGGTGCTGATGTCGATATTTAACAACGGGTGGAAC
CTGGGCTATCTGCACTGCCAAGTCAGTGGGTTCTGATGGGCCTGAGCGTCATCGGCTCC
ATATTCAACATCACCGGCATCGCCATCAACCGCTACTGCTACATCTGCCACAGTCTCAAG
TACGACAACTGTACAGCAGCAAGAACTCCCTCTGCTACGTGCTCCTCATATGGCTCCTG
ACGCTGGCGGGCGTCCTGCCAACCTCCGTGCAGGGACTCTCCAGTACGACCCGAGGATC
TACTCGTGACACCTTCGCCAGTCCGTGAGCTCCGCTACACCATCGCCGTGGTGGTTTTT
CACTTCCTCGTCCCATGATCATAGTCATCTTCTGTTACCTGAGAATATGGATCCTGGTT
CTCCAGGTGAGACAGAGGGTGAAACCTGACCGCAAACCCAACTGAAACCACAGGACTTC
AGGAATTTTGTACCATGTTTGTGGTTTTTGTCTTTTTGCCATTTGCTGGGCTCCTCTG
AACTTCATTGGCCTGGCCGTGGCCTCTGACCCGCCAGCATGGTGCCTAGGATCCCAGAG
TGGCTGTTTGTGGCCAGTTACTACATGGCGTATTTCAACAGCTGCCTCAATGCCATTATA
TACGGGCTACTGAACCAAAATTTAGGAAGGAATACAGGAGAATTATAGTCTCGCTCTGT
ACAGCCAGGGTGTTCTTTGTGGACAGCTCTAACGACGTGGCCGATAGGGTTAAATGGAAA
CCGTCTCCACTGATGACCAACAATAATGTAGTAAAGGTGGACTCCGTTTAAAAAGCACC
ACGTTCCGGGTGAGATGGACACGCT - 3'

```

Figure 6.12 (A) Map of the MT1 sequence and primer localization.

DNA from all 12 mel1a-3xHA-pcDNA3 minipreps was tested in a PCR reaction performed according to (see 5.10). PCR products were separated on a 2 % agarose gel as described in (see 5.11) and results are depicted in (Figure 6.13). In all samples, a PCR product of the expected size was amplified.

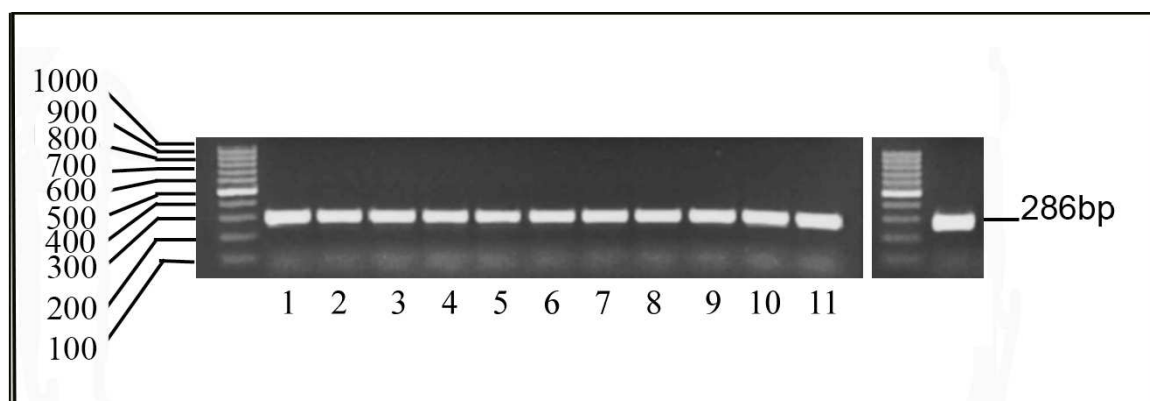


Figure 6.13 Detection of a 286 bp PCR product encoding parts of the MT1 melatonin receptor sequence.

Based on the above results, one of the minipreps (#7) was randomly chosen for further expansion. 100 ml of LB medium containing ampicillin were inoculated with 10 µl of the remaining bacteria suspension. Bacteria were expanded according to (see 5.7.5) and mel1a-3xHA-pcDNA3 DNA was purified using a purification kit (Pure Yield Plasmid Midiprep system, Promega). 400 µl of purified DNA were obtained in A. bid. DNA concentration (0.72 µg / µl) and purity (1.8) based on the absorption at 260 nm and 260 nm / 280 nm ratio, respectively were determined as described in (see 5.8).

In this sample, the sequence of the 3xHA-MT1 insert in pcDNA3 was verified by automated sequencing with the BigDye terminator principle using an AB 3100 Genetic analyser (Applied Biosystems in both directions using T7 and SP6 specific primers (DI Martin IBL, Austria)

6.2.2 Stable transfection of the eucaryotic BeWo cell line

Next, BeWo cells were stably transfected with the plasmid mel1a-3xHA-pcDNA3 using the calcium-phosphate transfection protocol according to (see 5.13). Selection of transfected cells was started 2 days after transfection by addition of 0.25 mg / ml G 418 (Geneticin) to the culture medium. Usually, mammalian cells are sensitive to the aminoglycoside antibiotic G418, which inhibits protein synthesis. However, pcDNA3 encodes for neomycin phosphotransferase thereby conferring resistance to G418 to transfected cells. The optimal concentrations of G418 for selection of stable transfected BeWo cells have been determined earlier in our lab [259, 406]. Following 17 – 19 days of selection, several G418-resistant BeWo cells colonies were picked as described in 5.13 and expanded.

In summary, the mammalian expression vector pcDNA3 containing the cDNA for MT1 including an N-terminal 3x HA-tag was amplified, purified and the identity of the sequence was confirmed. The choriocarcinoma cell line Bewo was stably transfected with the plasmid and several G418-resistant cell clones were picked for further analysis of their MT1 mRNA and protein expression levels.

6.3 Analysis of MT1 over expression

To analyse expression of MT1 transcripts in BeWo cells, total RNA was extracted from BeWo cells according to (see 5.14). The RNA concentration was determined by measuring the absorption at A_{260} (see 5.14.5). All RNA samples showed satisfactory purity (>1.7) as indicated by determination of the A_{260}/A_{280} ratio (see 5.14.5). Integrity and quality of RNA samples was judged following native agarose gel electrophoresis of all RNA samples by inspection of 28S and 18S rRNA bands (see 5.11). Synthesis of cDNA was carried out using same amounts of total RNA (2 μ g) and the high capacity cDNA reverse transcription kit (Applied Biosystems) as described in (see 5.15).

To detect the expression of the MT1 gene in the parental BeWo cell line, PCR reactions were performed according to (see 5.16) following the reverse transcription of the RNA. Two different sets of MT1 primer pairs were used [44, 400]. The sequence of these primers is shown in (see 5.10 and 5.16), respectively. In all experiments, amplification of the house-keeping gene β -actin was used as control reaction for intact RNA and cDNA. Primers for β -actin are shown in (see 5.16) and were selected according to [407]. The PCR products were analyzed by native agarose gel electrophoresis (see 5.11).

For a quantitative comparison of expression levels of MT1 mRNA in BeWo cell clones stably transfected with mel1a-3xHA-pcDNA3 with the parental cell line, isolated mRNAs were again reverse transcribed using Applied Biosystems high capacity cDNA reverse transcription Kit and the cDNA (10 – 50 ng) was then amplified using TaqMan® gene expression master mix and MTNR1A as well as HPRT TaqMan Gene Expression Assays in the Step One Plus Real Time PCR System (all Applied Biosystems) as detailed in (see 5.17). Relative mRNA expression levels of MT1 were then calculated by the DDCT (threshold cycle, [403]) method and normalized to the expression of the housekeeping gene HPRT.

Expression of MT1 mRNA in BeWo cells has been demonstrated [365, 44]. Using either this set of MT1 primers or an independent set of primers according to [400] that is shown in (see 5.10), endogenous expression of MT1 mRNA in BeWo and placental tissue was confirmed by RT-PCR in our laboratory (Stefanie Pseier and Isabella Ellinger, unpublished data). Figure 6.14 (kindly provided by Stefanie Pseier) shows an example for the endogenous expression of MT1 RNA in BeWo cells. HOS and MG63 cells served as positive controls for MT1 mRNA expression [389]. Data were obtained in RT-PCR experiments combining the “Lanoix” primers for “human MT1” and cDNAs derived from the cell lines BeWo, HOS and MG63. One PCR-product corresponding to the expected 442 bp MT1 fragment is found in all samples.

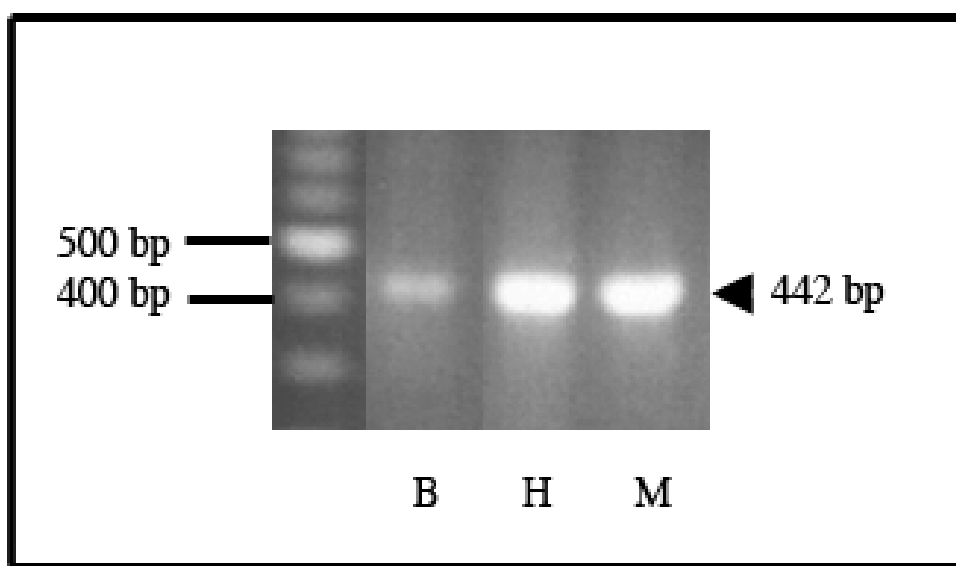


Figure 6.14 Detection of the MT1 transcript in (B) BeWo cells, and (H) HOS and (M) MG63 cells. Total mRNA was isolated, reverse transcribed and presence of MT1 transcripts was investigated by RT-PCR analysis using “MT1” primers described by [365, 44] (Data kindly provided by Stefanie Pseier)

For a quantitative comparison of the mRNA level in parental BeWo cells and BeWo cells stably transformed with mel1a-3xHA-pcDNA3, quantitative real time PCR was performed

using assays from Applied Biosystem for amplification of MT1 and HPRT as the reference gene. mRNA from the Ewing's sarcoma cell line TC71 [389] was included as a positive control as well as total human placental mRNA transcribed into cDNA. Results are shown in (Figure 6.15). Since endogenous MT1 mRNA in parental BeWo cells was under the limit of detection, expression of MT1 mRNA in the TC71 in relation to HPRT mRNA expression was chosen as a reference and set to 1. While expression of MT1 mRNA in total human placental extracts was only about 10 % of expression levels in TC71 cells, expression of MT1 mRNA in a variety of HA-MT1 transformed BeWo clones was 6 – 10-fold higher than expression in TC71 cells.

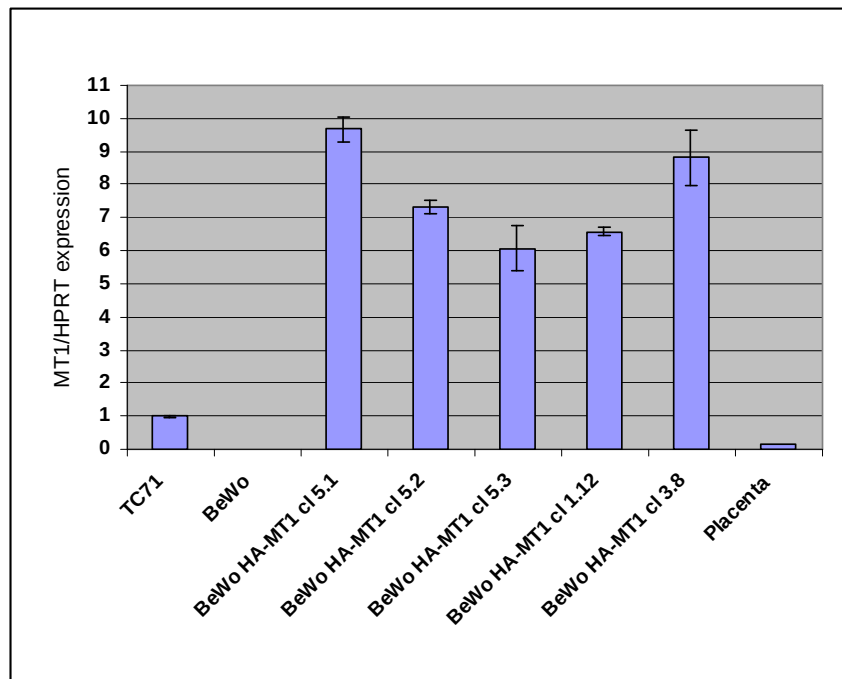


Figure 6.15 Quantitative comparison of MT1 transcripts in BeWo cells and BeWo clones stably transformed with mel1a-3xHA-pcDNA3.

Total mRNA was isolated, reverse transcribed and cDNA samples were analysed by TaqMan quantitative real time PCR for expression levels of MT1 and the housekeeping gene HPRT. Values of MT1 are normalized to the expression of HPRT.

To study expression of MT1 protein in BeWo cells stably transformed with MT1, cell homogenates were prepared according to (see 5.2) and the protein concentration was determined as described in (see 5.3). Same amounts of protein were separated by SDS-PAGE and transferred to PVDF-membranes as detailed in (see 5.4). MT1 expression was analysed using different sets of antibodies combining MT1- as well as HA-specific primary and HRP-labeled secondary antibodies (Set III-IV; see table 5.1) and detection of HRP-labeled proteins was performed as described in (see 5.4.6).

Following demonstration of increased MT1 mRNA expression in stably transformed BeWo cell clones, the anti-MT1 antibody sc-13179 (antibody set III, Table 5.1) was tested in western blot analysis of parental and MT1 (mRNA) overexpressing BeWo clones cell lysates (20 µg / lane) to detect (presumably) MT1 protein expression. As shown in Figure 6.16a, western blot analysis using anti-MT1 antibody SC-13179 revealed again 1 major band with a molecular weight of 40 kDa in all of the samples investigated. In Figure 6.16a, lysates containing identical amounts of protein of stably transformed clones 1.12, 5.1, 5.2 and 5.3 were analysed. According to Figure 6.15 these clones had much higher MT1 mRNA levels than the parental BeWo cell line. However, the sc-13179 antibody did not detect any significant increase compared to the level of protein expression in parental BeWo lysates. In contrast, the levels of protein expression detected by the antibody varied significantly. Moreover, the addition of a 3 x HA-tag to the MT1 protein should result in a small molecular weight increase of approximately 3 kDa (1xHA-tag = YPYDVPDYA=1102.15 Dalton) which should be observed in HA-MT1 overexpressing cells. Such an increase was not observed.

Finally, an identical blot was prepared but incubated with an anti-HA-tag antibody in order to detect HA-tagged MT1 receptor in the transformed BeWo cells. Results are shown in (Figure 6.16b). A variety of bands was detected by the antibody; however neither of them corresponded to the 40 kDa band detected by sc-13179. Most of the bands were detected in all samples loaded including parental BeWo cells and must therefore be assumed to be unspecific. This clearly asks for a further optimization of antibody dilution. However, a band of approximately 70 kDa (arrow in Figure 6.16b) was detected only in transformed BeWo cells, suggesting specific recognition of an HA-tagged protein in these cell lysates.

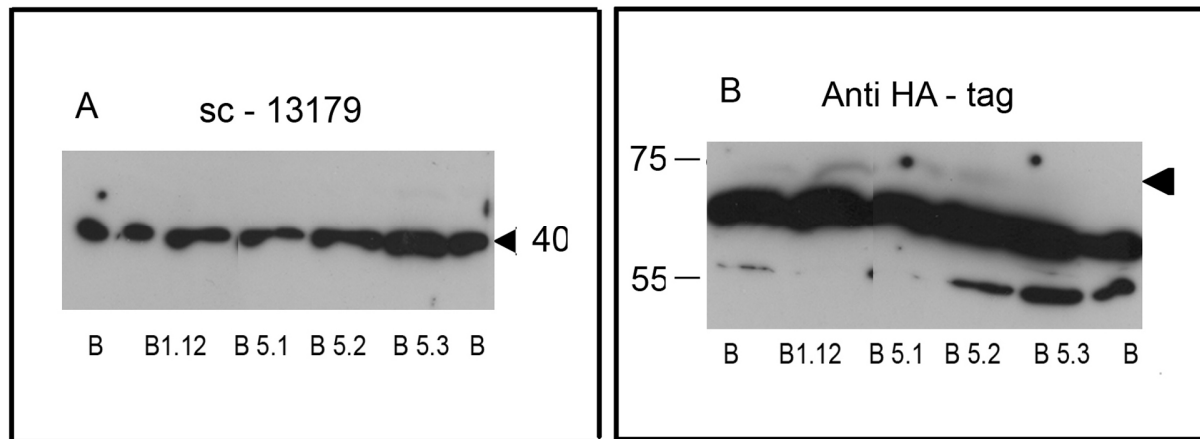


Figure 6.16 Western blot analysis of MT1 in (B) parental BeWo cells and a selection of HA-MT1 stably transformed BeWo cell clones(1.12, 5.1, 5.2, 5.3) by anti-MT1 antibody sc-13179 and an anti-HA- tag antibody.

Similar amounts of protein (20 µg) were separated by SDS-PAGE and transferred to PVDF membranes. Two identical membranes (a+b) were prepared. In (a), the membrane was incubated with "antibody set I" containing the primary antibody "anti-MT1 (sc-13179)", while in (b) the membrane was incubated with "antibody set IV" containing the primary antibody "anti-HA-tag (GeneTex GTX21424)". Numbers indicate the position for each protein marker in kilodalton. The arrow in (b) indicates a protein specifically recognized in HA-MT1 stably transformed BeWo cell clones.

In summary, several clones of BeWo cells stably transfected with the cDNA for HA-MT1 was demonstrated to express higher levels of MT1 mRNA than the parental cell line. Nevertheless, neither an increase in total expression of the 40 kDa protein nor an increase in molecular weight in these clones detected by the anti-MT antibody sc-13179 in western blotting experiments could be demonstrated. Preliminary results from an application of an anti-HA-tag antibody in western blotting experiments suggested the expression of an approximately 70 kDa HA-tagged protein in the transformed BeWo cells.

Taken together, these data suggest that the anti MT1 antibody sc-13179 recognizes a 40 kDa protein corresponding (based on the molecular weight) to either a non-glycosylated form of the MT1 receptor or an unknown protein that shares sequence homology with MT1. Since N-glycosylation sites of endogenous MT1 and HA-tag of transfected MT1 are localized in the proximity of the N-terminal sequence recognized by sc-13179, glycosylated and HA-tagged MT1 might not be recognized by the antibody. Based on the preliminary results obtained with the anti-HA-tag antibody, a HA-tagged protein (HA-MT1) of about 70 kDa might be expressed in the transfected cells. In conclusion, sc-13179 is not a reliable antibody for detection of mature human MT1 receptor.

7 DISCUSSION

Several studies suggested so far that MT via receptor-mediated actions plays an important role in the regulation of human trophoblast development (e.g. proliferation, differentiation, and apoptosis) and endocrine functions (e.g. hormone production; [364, 149, 365, 382, 383]). However, the receptor-mediated effects of MT in placental chorionic cells have not been investigated systematically. Therefore, *in vitro* models to study expression and function of human MT receptors in human chorionic trophoblast cell lines need to be established. The choriocarcinoma cell line BeWo is derived from human trophoblast cells and has often been used to study trophoblast functions [257, 258, 259, 260, 261, 262, 263]. BeWo cells were found to express MT1 mRNA in this study, which is in good agreement with the results of other groups [365, 44].

Investigations of MT receptors at the protein level are, however, often limited by a lack of well-characterized anti-MT1 antibodies.

Over the years, the expression and localization of the human MT1 receptor protein has been investigated using various anti-MT1 antibodies. The best characterized antibody has been raised against a peptid corresponding to 19 amino acids of the c-terminus of the MT1 [177]. This antibody was characterised in COS and HEK 293 cells transfected with the MT1 cDNA and was found to recognize a 60 kDa protein in the lysates of membrane fractions. G-protein coupled receptors are highly hydrophobic and nearly always glycosylated. Indeed, upon deglycosylation, an additional protein with a molecular weight of 40 kDa was detected by Brydon and coworkers by their antibody [177]. Though this antibody has been used in several subsequent studies dealing with expression and localization of MT1 in human tissues [163, 408, 160, 161, 165], it is unfortunately unavailable for general disposal.

Some commercially available antibodies have also been used for demonstration of human MT1 expression by western blotting and/or immunofluorescence microscopy. A frequently used commercial antibody was an anti-MT1 antibody from former CIDtech Research Inc. (now Chemiclones Inc.) that depending of the cell line or tissue under investigation reacted with various bands in the range between 37 kDa and 50 kDa [409, 410, 411, 164, 412, 413]. This antibody has never been completely characterized, and, anyway, is no longer sold.

To study human MT1 protein function in BeWo cells, three commercially available anti-MT1 antibodies were chosen in this study and their applicability in western blotting and immunofluorescence microscopy was tested. Two of these antibodies were obtained from Abcam Company. The epitopes of ab-13035 and ab-13036 are localized in the 2nd and 3rd intracellular loop of human MT1, respectively. To our knowledge, the only published

application of ab-13036 is an immunofluorescence microscopic study demonstrating vesicular cytoplasmic localisation of MT1 in epithelial cells of the human breast [405]. The third antibody was sc-13179 obtained from the Santa Cruz Company that binds to the N-terminus of human MT1 and has been used to show MT1 expression in human cell lines and tissue by western blotting and immunofluorescence microscopy [365, 44, 404]. In BeWo cells and placental tissue extracts, sc-13179 was shown to detect a protein of approximately 40 kDa by western blotting [365, 44]. In immunofluorescence microscopy the antibody revealed a predominant intracellular localization of MT1 in BeWo cells [365]. Investigating MT1 expression in the human prostate epithelial cell lines RWPE-1 and 22Rv1 cells, Tam and coworkers [404] applied sc-13179 in western blotting experiments and also demonstrated only 1 reactive band; unfortunately, the molecular weight of this band was not indicated in this study.

In this study, in a first set of experiments, we tested the capability of these three antibodies to detect endogenous MT1 protein in BeWo cells. Results obtained by immunofluorescence microscopy were similar for all three antibodies, demonstrating mainly an intracellular, vesicular localisation of the target, presumably MT1. A predominant intracellular localisation of MT1 in BeWo cells is in good agreement with the studies conducted by Lanoix and coworkers [365] using sc-13179. Likewise, MT1 expression in human breast cells investigated by Ab-13036 [405] was found to be mainly intracellular. The antibody generated by Brydon and coworkers [177] localised MT1 to granular structures in the cytoplasm of neurons [302]. Finally, upon expression of HA-tagged MT1 in CHO cells, the majority of MT1 localized to intracellular compartments [397].

In contrast, the results obtained by western blotting were more heterogenous. The Abcam antibodies ab-13035 and ab-13036 detected several bands with a broad range of molecular weight (40 – 130 kDa), however, shortly after these initial studies, these antibodies became commercially unavailable; consequently, we focused our further investigations on sc-13179. In our hands, sc-13179 reproducibly recognized only one major band with a molecular weight of 40 kDa in BeWo cells and placental tissue lysates, in good agreement with Lanoix et al. [44]. The antibody also detected a protein of identical size in other cell lines with known expression of MT1 mRNA, such as the ovarian cancer cell line OVCAR-3 [461] and the human osteosarcoma cell lines HOS and MG63 [389]. MT1 has indeed a predicted molecular mass of approximately 40 kDa, however, for the unglycosylated core protein [54]. As indicated above, G-protein coupled receptors are nearly always glycosylated [414, 415] and likewise the presence of two potential glycosylation sites in the N-terminal domain of the human MT1 receptor would suggest receptor glycosylation. A glycosylated receptor, however, would migrate with a molecular mass greater than that predicted from the amino

acid sequence. Detection of a 40 kDa protein in BeWo cells (and human placenta) therefore suggests that BeWo cells only express a non-glycosylated MT1 receptor. Alternatively, the glycosylated receptor might not be detected by sc-13179. This second scenario could be explained by the proximity of the N-glycosylation sites of MT1 and the target sequence of sc-13179 (see figure 6.1). A third option is that sc-13179 does not detect MT1 at all, but recognizes a protein that shares sequence homologies with MT1.

To be able to differentiate between these possibilities and further characterize the specificity of the sc-13179 antibody, BeWo cells were stably transfected with the cDNA for a HA-tagged human MT1 receptor. Though we were able to demonstrate overexpression of MT1 mRNA in several clones, the size and the amount of protein detected by sc-13179 were not significantly altered in comparison to the parental cell line. This result can be interpreted in two ways. 1/ Overexpressed HA-MT1 were not translated into protein. However, in preliminary studies using an anti-HA-tag antibody, we detected a ~70 kDa protein in the transfected clones but not the parental cell line, suggesting that at least in some clones an HA-tagged MT1 was expressed. The molecular weight (70 kDa) suggests that this HA-MT1 is presumably a glycosylated protein. Nevertheless, more studies are required to confirm these results and better characterize the anti-HA-tag antibody. 2/ The N-terminal HA-tagged MT1 receptor was not recognized by sc-13179 due to sterical hindrance similar to the endogenous glycosylated receptor and consequently the sc-13179 either only detected the non-glycosylated (endogenous) form of the receptor or reacted with an unknown protein sharing sequence homologies with MT1. In summary, the identity of the protein identified by sc-13179 in western blotting experiments remains obscure and the usability of sc-13179 to characterize MT1 function in placental trophoblast cells is still questionable.

8 CONCLUSIONS

This study aimed to better characterize three anti-human MT1 antibodies. Two of the antibodies investigated are no longer available. Sc-13179 (Santa Cruz) was found to react positively in western blotting experiments and immunofluorescence microscopy with cell lines and tissues, where expression of endogenous MT1 mRNA has been demonstrated. Despite generation of cell lines with increased levels of MT1 mRNA ("HA-tagged-MT1-overexpressing cells"), the identity of the 40 kDa protein detected by the antibody remains obscure. Most likely, sc-13179 detects a non-glycosylated MT1 receptor or a yet unknown protein sharing sequence homologies with MT1 in the antibody binding site. Though more work is needed to characterize the nature (molecular weight, glycosylation state) of the endogenous MT1 in BeWo cells as well as the overexpressed HA-tagged MT1 protein, our results obtained so far suggest that sc-13179 is not suitable to investigate the functions of the fully processed MT1 receptor in human trophoblast cells.

9 LIST OF ABBREVIATIONS

AAAD	Aromatic -1- amino acid decarboxylase
AANAT	Arylalkylamine-N-acetyltransferase
A.bid.	aqua bidestillata
AD	Alzheimer's disease
AFMK	N ¹ -acetyl-N ² -formyl-5-methoxykynuramine
AMK	N1-acetyl-5-methoxykynuramine
AMPS	ammoniumpersulfate
BSA	bovine serum albumin
Ca ⁺⁺	calcium
CTB	cytotrophoblasts
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
EtBr	ethidium bromide
FCS	fetal calf serum
g	gram
<i>g</i>	gravity
x <i>g</i>	amount of acceleration quoted in multiples of <i>g</i>
h	hour(s)
HAc	acetic acid
HIOMAT	Hydroxyindole-O-methyltransferase
HRP	horseradish peroxidase
HSA	human serum albumin
K ⁺ channels	Kir channels
MeOH	methanol
min	minute(s)
MM	master mix
MT	Melatonin
5 - MT	5-methoxytryptophol
NE	nor epinephrine
nNOS	neuronal NO synthase
on	over night
OS	original sample
PA	polyacrylamide

List of Abbreviations ;continue

PBS	phosphate buffered saline
pc	protein concentration
PD	Parkinson's disease
PE	preeclampsia
PFA	paraformaldehyde
PH	Power of hydrogen
PKA	protein kinase A
PKC	Protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulphonyl fluoride
PSN	Liquid antibiotic mixture containing <u>P</u> enicillin, <u>S</u> treptomycin and <u>N</u> eomycin
PVDF	poly-1,1-difluoroethene or polyvinylidene difluoride
QR2	quinone reductase 2
rt	room temperature
ROS	reactive oxygen species
rpm	revolutions per minute (unit of frequency to measure rotational speed)
SB	sample buffer
SCN	suprachiasmatic nuclei
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
sec	second(s)
STB	syncytiotrophoblast
sup	supernatant
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
T5 M	Tryptophan-5-monooxygenase
Tris	tris(hydroxymethyl)-aminomethane
TX-100	triton X 100
w/v	weight per volume
v/v	volume per volume

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