Fall 2010

Effect of Melatonin on Differentiation of Human Mesenchymal Stem Cells and a Study on C-Terminal Domains of MT1 and MT2 Melatonin Receptors

Shalini Sethi

EFFECT OF MELATONIN ON DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS AND A STUDY ON C-TERMINAL DOMAINS OF MT_1 AND MT_2 MELATONIN RECEPTORS

A Dissertation

Submitted to the Graduate School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

By

Shalini Sethi

December 2010

EFFECT OF MELATONIN ON DIFFERENTIATION OF HUMAN

MESENCHYMAL STEM CELLS

AND

A STUDY ON C-TERMINAL DOMAINS OF MT_1 AND MT_2 MELATONIN

RECEPTORS

By

Shalini Sethi

Approved June 23, 2010

Paula A. Witt-Enderby, Ph.D.
Thesis Chairperson
Professor of Pharmacology and Toxicology
Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA

Vicki L. Davis, Ph.D.
Assistant Professor of Pharmacology
Graduate School of Pharmaceutical Sciences
Duquesne University
Pittsburgh, PA

Jane E. Cavanaugh, Ph.D.
Assistant Professor of Pharmacology
Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA

John A. Pollock, Ph.D.
Associate Professor
Department of Biological Sciences
Bayer School of Natural and Environmental Sciences
Duquesne University, Pittsburgh, PA

Rehana K. Leak, Ph.D.
Assistant Professor of Pharmacology
Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA

J. Douglas Bricker, Ph.D.
Dean
Mylan School of Pharmacy and the Graduate School of Pharmaceutical Sciences
Duquesne University, Pittsburgh, PA

EFFECT OF MELATONIN ON DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

AND

A STUDY ON C-TERMINAL DOMAINS OF MT_1 AND MT_2 MELATONIN RECEPTORS

By

Shalini Sethi

June 2010

Dissertation supervised by Paula A. Witt-Enderby, Ph.D.

Abstract 1: Melatonin has been reported to enhance the differentiation of osteoblasts. The purpose of this study was to determine the melatonin treatment that would differentiate human mesenchymal stem cells (hAMSCs) into osteoblasts. A 21 d continuous melatonin treatment significantly increased the alkaline phosphatase (ALP) activity and the deposition of calcium in hAMSCs. These effects were inhibited by MT_2 specific antagonist- 4P-PDOT. The time periods of melatonin treatment that increased the expression of osteogenic genes indicated both a sensitized and desensitized receptors with respect to cAMP signaling, signifying two distinct mechanisms of melatonin's action. Unlike the parathyroid hormone which is

administered in intermittent doses to increase bone mass, a continuous melatonin treatment may be effective in having an anabolic effect on bone.

Abstract 2: In this study we sought to understand the domains involved in the function and desensitization of MT_1 and MT_2 receptors through site-directed mutagenesis. Two mutations were constructed in the cytoplasmic C-terminal tail of each receptor subtype- 1) A putative palmitoylation site (a cysteine residue) in the C-terminal tail was mutated to alanine (MT_1C7.72A and MT_2C7.77A) and 2) the C-terminal tail in the was truncated (MT_1Y7.64 and MT_2Y7.64). Through confocal microscopy, it was determined that the putative palmitoylation site did not play a role in receptor internalization, however, this residue was essential for receptor function, as determined by cAMP accumulation assays. Truncation of the C-terminal tail of both receptors inhibited internalization as well as the cyclic AMP response, suggesting the importance of the C-terminal tail in these receptor functions.

DEDICATION

There are not enough words to acknowledge the professional, scientific and personal support that I have received from my advisor Prof. Paula Witt-Enderby. Her scientific input, guidance and enthusiasm for research were indispensable to completing this work and understanding the various aspects of this project. She is the most positive person I have met and her constant optimism and encouragement has been key to shaping my outlook towards research and life in general.

I would like to express sincere gratitude to my committee members for their valuable contribution to this dissertation. Other than providing constant support for this project as a committee, I would like to thank Dr. Vicki Davis for her help with RT-PCR studies and immense guidance with presenting scientific data, Dr. John Pollock for his guidance with confocal microscopy and helping me look at the data from various angles, Dr. Jane Cavanaugh for the use of her laboratory space, her assistance with western blot assays and teaching me critical thinking on scientific articles and Dr. Rehana Leak for agreeing to be on this committee on short notice and contributing her valuable time to this dissertation.

I would like to thank Dr. Yau-Huei Wei and Chein-Tsun Chen from National Yang –Ming University, Taiwan for their collaboration on this project and Dr. Christopher Surratt for his training with scientific writing and providing his input on this dissertation.

I would like to acknowledge my colleagues Mary Kotlarczyk for her assistance with designing primers for RT-PCR experiments and Dr. Wendy Adams for her help with mutagenesis experiments.

Thanks are also due to my colleagues who have provided technical and personal support during my time at Duquesne University- Dr. Nicholas Radio, Erika Allen, Bill Clafshenkel, Bala Sunder Reddy, Pangi Johnson and Rachel Kirker.

Lastly, I would like to thank my husband Shringi and my parents for their unwavering support and patience through all these years. Their confidence in my abilities has been highly instrumental in reaching this stage of life.

TABLE OF CONTENTS

Page

Chapter 1
Treatment Effects of Melatonin on Differentiation of Human Mesenchymal Stem Cells into Osteoblasts

1.INTRODUCTION 1
1.1 Bone and Osteoporosis 1
1.2 Melatonin Synthesis 2
1.3 Mechanisms of Action of Melatonin in Cells 5
1.3.1 Receptor Dependent Actions of Melatonin....5
1.3.2 Receptor Independent Actions of Melatonin 6
1.4 Melatonin and Bone 7
1.4.1 Human and Animal Studies 8
1.4.2 In- Vitro Studies....10
1.5 Signaling Events Governing Osteoblast Differentiation 12
1.6 Mechanisms of Action of Melatonin on Osteogenesis....17
1.7 Human Adult Mesenchymal Stem Cell Model 22

2.MATERIALS AND METHODS 23
2.1 Cell Culture 23
2.2 Treatment Groups....24
2.3 Alkaline Phosphatase Assay....27
2.4 Quantitative Analysis of Osteogenesis....27
2.5 Qualitative Analysis of Osteogenesis....28
2.6 Radioligand Binding Assays 28
2.7 cAMP Accumulation Assays 29
2.8 Cell proliferation Assays 30
2.9 RNA Extraction....30
2.10 RT-PCR Reactions 31
2.11 Real-Time RT-PCR....31
2.12 Statistical Analysis 36

3.RESULTS 37
3.1 The Effect of Melatonin on ALP Activity in hAMSCs 37
3.2 Melatonin Levels Expression Levels in hAMSCs Exposed to Melatonin over Different Periods of Time 40
3.3 Saturation Binding Analysis on hAMSCs Exposed to Melatonin over Different Time Periods....42
3.4 Functional Analysis of Melatonin Receptors Expressed in Differentiating hAMSCs Exposed to Melatonin over Different Time Periods. 44
3.5 The Effect of Melatonin Exposure on Calcium Deposition in Differentiating hAMSCs 46
3.6 Qualitative Assessment of Calcium Deposition in Differentiating hAMSCs Exposed to Melatonin for 21 d 49

3.7 Proliferation of hAMSCs Treated with Melatonin for Different Times.... 51
3.8 The Effect of a 21 d Melatonin Exposure on Mitochondrial Gene Expression in Differentiating hAMSCs 52
3.9 The Effect of a 21 d Melatonin Exposure on Genes Involved in Anaerobic Metabolism 54
3.10 The Effect of Various Melatonin Exposures on Expression of Genes Involved in Osteogenesis 56

4.DISCUSSION 59

5.CONCLUSION 74

Chapter 2
A Study on C-Terminal Domains of MT_1 AND MT_2 Melatonin Receptors

6.INTRODUCTION 75

7.MATERIALS AND METHODS 79
7.1 Cell Culture and Transfections....79
7.2 Construction of Mutant Plasmids....79
7.3 Radioligand Binding Assays 80
7.4 cAMP Accumulation Assays 81
7.5 Confocal Microscopy 81
7.6 Radiolabeling with [^{3}H] Palmitic Acid 82

8.RESULTS 84
8.1 Effect of the C-terminal mutations on Affinity and Density of 2-[^{125}I]-Iodomelatonin Binding to MT_1 and MT_2 Melatonin Receptors Expressed in COS-7 cells....84
8.2 Effect of C-terminal Mutations on Melatonin-Mediated Inhibition of Forskolin-Induced cAMP Accumulation in Naive COS-7 cells Expressing MT_1 and MT_2 Receptors 85
8.3 Effect of the C-terminal Mutations on Melatonin-Mediated Inhibition of Forskolin-Induced cAMP Accumulation in COS-7 cells Expressing MT_1 and MT_2 Receptors that were Pretreated with 1 μM Melatonin....87
8.4 Effect of the C-terminal Mutations on the Internalization Patterns of MT_1 and MT_2 Receptors Expressed in COS-7 Cells following Melatonin Exposure....89
8.5 Effect of Palmitoylation on WT MT_1 and MT_2 Receptors....91

9.DISCUSSION 92

10.CONCLUSION 96

11.REFERENCES 97

12.APPENDIX 115
12.1 Sample Calculations for measuring ALP Activity in hAMSCs 115
12.2 Sample Calculations for Quantitative Analysis of Osteogenesis 118
12.3 Sample Calculations for Real- Time RT- PCR 120

LIST OF FIGURES

Figure 1: Fluctuations in Melatonin Levels over a 24 h Period in humans 4
Figure 2: Diagrammatical Representation of the Different Stages of Differentiation of human Mesenchymal Stem cells into Osteocytes 13
Figure 3: Important Signaling Mechanisms Involved in Osteoblast Differentiation..16
Figure 4: Schematic Representing Melatonin's Effects on Osteoclasts 18
Figure 5: The Study Design 25
Figure 6: The Study Design for Investigating the Effect of Varying the Duration of OS+ Medium on Melatonin- Induced Osteoblast Differentiation 26
Figure 7: The Effect of Different Exposure Times on hAMSCs Differentiation into Osteoblasts 39
Figure 8: The Effect of Varying Pre-exposure to Osteogenic (OS+) Medium on 2 d Melatonin Induction of ALP in Differentiated hAMSCS 41
Figure 9: Total 2-[^{125}I]-iodomelatonin Binding Analysis on hAMSCs Treated with Melatonin over Different Time Periods 43
Figure 10: Functional Analysis of Melatonin Receptors Expressed in hAMSCs Exposed to Melatonin over Different Time Periods 45
Figure 11: The Effect of Melatonin Exposures on Calcium Deposition in Differentiating hAMSCs 47
Figure 12: Qualitative Assessment of Calcium Deposition in Differentiating hAMSCs Exposed to Melatonin using Alizarin Red Staining 50
Figure 13: Proliferation of hAMSCs Treated with Melatonin for Different Times ...51
Figure 14: The Effect of a 21 d Melatonin Exposure on Mitochondrial Gene Expression in Differentiating hAMSCs 53
Figure 15: The Effect of a 21 d Melatonin Exposure on Genes Involved in Anaerobic Metabolism 55
Figure 16: The Effect of Melatonin Exposure on the Expression of Genes Involved in Osteogenesis 58
Figure 17: Proposed Mechanism of Melatonin's Action within a Differentiating Human Adult Mesenchymal Stem Cell into an Osteoblast 72
Figure 18: Details of the C-terminal Mutations 83
Figure 19: Melatonin Mediated Inhibition of Forskolin-induced cAMP Accumulation in COS-7 cells Transiently Expressing WT or Mutant Melatonin Receptors 86
Figure 20: Melatonin Mediated Inhibition of Forskolin Induced cAMP Accumulation in Melatonin-Pretreated COS-7 cells Transiently Expressing WT or Mutant Melatonin Receptors 88
Figure 21: Internalization Patterns of Green Fluorescent Protein-Tagged WT and Mutant Receptors Following Exposure to Melatonin 90
Figure 22: Incorporation of 9, 10-[3H] palmitic acid in COS-7 Cells Transiently Transfected with WT MT_1 and WT MT_2 Receptors 91
Figure 23: Standard Curve Depicting the Rate of Hydrolysis of p-Nitrophenyl Phosphate Disodium Hexahydrate 115
Figure 24: Standard Curve Depicting Total Protein 116
Figure 25: Standard Curve Depicting Colorimetric Analysis of Known Concentrations of Alizarin Red Solutions 119

Figure 26: Sample Real-time PCR Graph Depicting C_t values for GAPDH and Gene of Interest 120
Figure 27: Logarithmic Graph View of Example Real-Time PCR data.....121
Figure 28: Sample Real-Time PCR Melt Curve.....122

LIST OF TABLES

Table 1: Primers for Real-Time RT-PCR 35
Table 2: Saturation Binding Analysis of 2-[^{125}I]-Iodomelatonin Binding to Mutant and WT MT_1 and MT_2 Receptors 84
Table 3: Standard Curve Values Depicting the Rate of Hydrolysis of p-Nitrophenyl Phosphate Disodium Hexahydrate 115
Table 4: Standard Curve Values Depicting Total Protein via BSA Assay 116
Table 5: Sample ALP Activity Calculation of Control vs. 21 d Melatonin Exposure 117
Table 6: Standard Curve Values Depicting Colorimetric Analysis of Known Concentrations of Alizarin Red Solutions 118
Table 7: Sample Calculation for Quantitative Analysis of Osteogenesis of Control vs. 21 d Melatonin Exposure 119
Table 8: Calculation of Fold Change in Gene Expression with 21 d Melatonin Exposure over Control 122

Chapter 1
Treatment Effects of Melatonin on Differentiation of Human Mesenchymal Stem Cells into Osteoblasts

1. INTRODUCTION

1.1 Bone and Osteoporosis

Bone remodeling is a constant and dynamic process. The cells involved in bone metabolism are of two types- osteoclasts that resorb old bone, and osteoblasts that form new bone. Together these cells form the 'bone metabolism unit'. The formation of new bone and the resorption of old bone are tightly coupled to each other. Bone remodeling is initiated by osteoclasts which attach to old bone and erode the bone matrix. Once the osteoclasts leave the unit, osteoblasts are recruited to this site and they start to fill up the cavities formed by the osteoclasts, thus forming new bone in the process (Christenson, 1997; Manolagas, 2000).

An imbalance between the osteoclasts and osteoblasts equilibrium can lead to osteoporosis, a disease characterized by a decrease in bone density and an increase in risk of fractures. Approximately 10 million Americans have osteoporosis, out of which 80% are women . One of the primary causes of osteoporosis in women is estrogen deficiency with age or menopause (Lindsay et al., 1976; Horsman et al., 1977; Lufkin and Ory, 1989; Christiansen, 1993).

Most treatments for osteoporosis aim at preventing further bone loss by inhibiting the activity of osteoclasts by use of bisphosphonates, calcitonin, selective estrogen receptor modulators and supplementing one's diet with calcium and/ or vitamin D. (Delmas, 2002; Uebelhart et al., 2003; Ishida and Kawai, 2004). These therapies have

anti-resorptive mechanisms and as such, they do not increase bone mineral density. As the scope of these therapies is limited by their inability to form new bone, there is a need to discover newer therapies that will have anabolic effects on the bone. The only FDA approved treatment that can build new bone by targeting the proliferation, differentiation, and activity of osteoblasts is teriparatide PTH 1-34 (recombinant human 1-34 parathyroid hormone; brand name: FORTEO) (Rosen and Bilezikian, 2001; Brixen et al., 2004). This treatment requires daily subcutaneous injections of the peptide for many years.

A decrease in the hormone melatonin in serum with age has been related to increase in risk of osteoporosis (Sandyk et al., 1992; Ostrowska et al., 2001b; Cardinali et al., 2003). Melatonin has been shown to enhance differentiation of osteoblasts in various cell models and it could prove to be a novel anabolic therapy for osteoporosis (Roth et al., 1999; Radio et al., 2006; Satomura et al., 2007). Also, melatonin has been shown to inhibit osteoclast activity (Suzuki and Hattori, 2002). The objective of this study was to determine the critical time periods of melatonin exposure that cause differentiation of osteoblasts and to study the various genes that are involved in this process.

1.2 Melatonin Synthesis

Melatonin is a hormone released from the pineal gland in response to darkness. Melatonin concentrations vary in different body fluids, in different cells, in different sub cellular organelles and in different organisms (Reiter and Tan, 2003). Melatonin production in the body varies with the time of the day, with highest production being in darkness. The levels of melatonin persist for longer periods of time during the winter months (Witt-Enderby et al., 2003). In healthy human subjects, the average maximum levels attained in the plasma of adults are around of 60 to 70 pg/ml. The levels begin

increasing around 9-10 pm and remain high till 7-9 am. Peak concentrations are attained between 2 am to 4 am (Zawilska et al., 2009).

Melatonin is synthesized in the pineal gland from serotonin. Serotonin is converted to N- acetyl serotonin by the action of enzyme serotonin N-acetyl transferase. N-acetyl serotonin is acted on by hydroxyindole *O*-methyltransferase to form melatonin (Hickman et al., 1999; Schomerus and Korf, 2005). N-acetyl transferase is the key regulatory enzyme in this biosynthetic pathway and changes in its levels reflect changes in melatonin production (Zawilska et al., 2009). The activity of this enzyme in mammals in controlled by a master pacemaker circadian clock located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus (Weaver, 1998; Reppert and Weaver, 2002).

Light is the most important signal that regulates the activity of N-acetyl transferase and consequently melatonin production and release in the body. In mammals, the pineal gland itself is not photosensitive, but it can 'sense' the light and darkness through a multi-synaptic pathway that connects the SCN with the pineal gland (Korf et al., 1998). The final part of this pathway consists of sympathetic nerve fibers that innervate the pineal parenchyma and release norepinephrine (NE) in a circadian manner at night. SCN receives the photic information from the retina resulting in the release of NE from the nerve fibers to bind the α_1 and β_1-adrenergic receptors on the pinealocytes. The activation of α_1 receptors lead to increases in intracellular calcium concentration whereas the activation of β_1 receptors lead to increases in intracellular cyclic AMP and protein kinase A. Both these changes cause phosphorylation of transcription factors CREB (cAMP regulatory element-binding protein) which induces the transcriptional production of *N*-acetyltransferase enzyme (Romero et al., 1975; Chansard et al., 2005;

Schomerus and Korf, 2005). Thus, the synthesis of N-acetyl transferase is regulated by the light/ dark cycle.

The N-acetyl transferase production is also influenced by circadian rhythms, independent of lighting conditions (Schwartz et al., 2009). The clock genes *Per1* and *Per2* and two mammalian *cryptochrome* (*Cry*) genes, *Cry1* and *Cry2* in the rat pineal gland are expressed in circadian fashion and are responsible for the self-sustaining rhythm generating capacity of the SCN, in the absence of light (Lincoln et al., 2002).

The nocturnal release pattern of melatonin is the result of both circadian control and light-inhibition of its synthesis (Fig. 1).

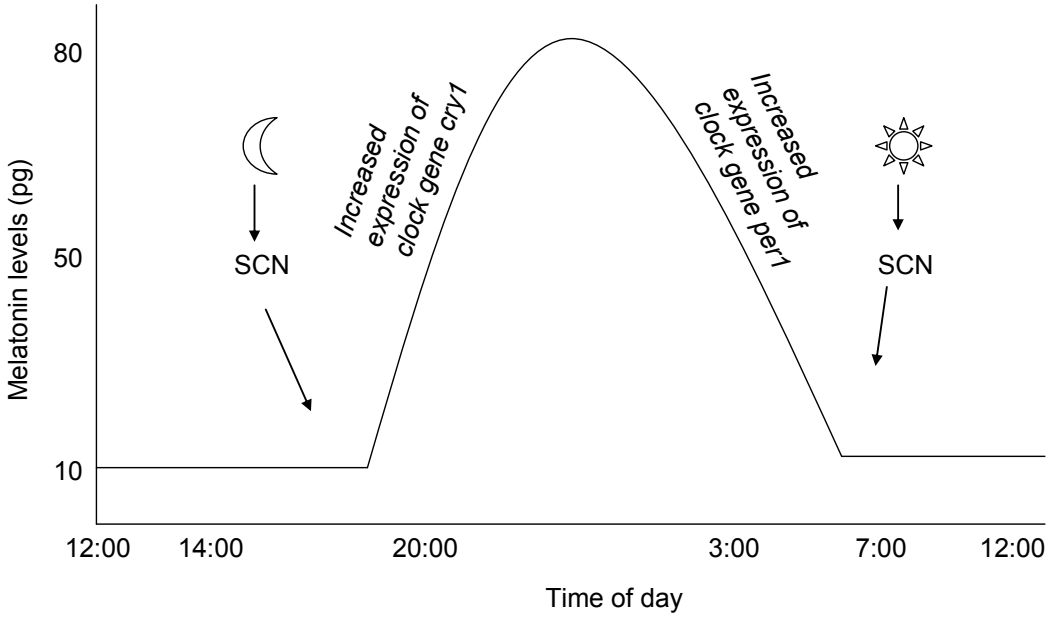


Figure 1: Fluctuations in Melatonin Levels over a 24 h Period in humans

The average maximum levels attained in the plasma of adults are around of 60 to 70 pg/ml. The levels begin increasing around 9-10 pm and remain high till 7-9 am. Peak concentrations are attained between 2 am to 4 am. The release pattern of melatonin is the result of both circadian control and light exposure, as explained in text.

1.3 Mechanisms of Action of Melatonin in Cells

1.3.1 Receptor Dependent Actions of Melatonin

Melatonin may bind to G-protein coupled receptors designated as MT_1 and MT_2 receptors or the binding site belonging to the quinone reductase 2 family of detoxifying enzymes, designated as MT_3 receptor (Nosjean et al., 2000; Delagrange et al., 2003; Witt-Enderby et al., 2006). Both MT_1 and MT_2 receptors are coupled mainly to G_i proteins that upon activation cause an inhibition of adenylyl cyclase enzyme in the cells (Grant et al., 2009). This decrease in second messenger cyclic AMP (cAMP) levels, PKA levels and CREB phosphorylation (McNulty et al., 1994; Witt-Enderby et al., 2003). The activation of G_i proteins can cause the dissociation of the $G_{i\alpha\beta\gamma}$ into $G_{i\alpha}$-GTP and $G_{i\beta\gamma}$. $Gi_{\beta\gamma}$ may transactivate the epidermal growth factor receptors (EGFR) and the activation of downstream effectors MEK and ERK. Activation of G_i may also result in the internalization of the receptors through binding of β-arrestin-2 and subsequent activation of MEKs and ERKs. The activation of the MAPK pathway has been linked to an increase in cell differentiation (Ahmed et al., 2003; Radio et al., 2006; Bondi et al., 2008; Grant et al., 2009).

MT_1 and MT_2 receptors are also coupled to G_q proteins, that upon agonist binding causes an activation of phospholipase C, which hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to form inositol 1, 4, 5-trisphosphate/ Ca^{2+} and diacylglycerol (Brydon et al., 1999; MacKenzie et al., 2002). Activation of MT_1 receptors may inhibit the formation of early gene products- c-fos and c-jun (Ross et al., 1996). The MT_2 melatonin receptor, in addition to coupling with G_i or G_q proteins, is able to inhibit the soluble guanylyl cyclase pathway (Petit et al., 1999).

The MT_1 melatonin receptor is expressed in the suprachiasmatic nucleus of the hypothalamus, pars tuberalis, retina and cardiac vessels (Witt-Enderby et al., 2003). MT_2 receptors are expressed in the retina, hippocampus, SCN, and cerebellum (Dubocovich et al., 2003; Alarma-Estrany and Pintor, 2007). MT_3 binding site is present in a wide variety of mammals and tissue, with highest receptor amounts and activity detected in the liver and kidney (Nosjean et al., 2001).

1.3.2 Receptor Independent Actions of Melatonin

Melatonin is capable of acting independently of the melatonin receptors. Melatonin is highly lipophilic and able to traverse the plasma membranes of the cells and bind to cytosolic proteins like calmodulin to regulate Ca^{2+} signaling pathways (Benitez-King et al., 1993; Dai et al., 2002). Melatonin inhibits calmodulin activity by preventing calcium binding, and causes a decrease in adenynlyl cyclase activity to decrease CREB protein activity and cAMP response element (CRE)-containing genes. Besides attenuating CRE- containing genes, melatonin, through an inhibition of calmodulin dependent adenylyl cyclases, can inhibit ERE- containing genes by inhibiting the binding of estrogen receptors (ER-α) to estrogen response elements. Melatonin can also down regulate estrogen synthesis by decreasing the release of gonadotropins (Roy et al., 2001) or by inhibiting aromatases (Molis et al., 1994; Rato et al., 1999; Witt-Enderby et al., 2003; Grant et al., 2009). These anti-estrogenic mechanisms suggest that melatonin may have an important role in bone metabolism, since estrogen is critical for maintaining bone mass in women. Estrogen modulates osteoclast activity and a loss of estrogen in body, either through menopause or tamoxifen therapy increases the risk of osteoporosis (Srivastava et al., 2001; Saintier et al., 2006; Robinson et al., 2009).

Besides the actions listed above, melatonin is also a direct free radical scavenger. It can directly detoxify free radical species, increase the activity of anti-oxidative enzymes and increase efficiency of mitochondrial oxidative phosphorylation to improve mitochondrial metabolism i.e., by avoiding electron leakage, enhancing complex I and complex IV activities, or by having a synergizing effect with other antioxidants (Reiter et al., 2003; Hardeland and Pandi-Perumal, 2005; Grant et al., 2009). The by- products generated by the free radical scavenging effects of melatonin are also free radical scavengers themselves. This radical scavenger on radical scavenger effect makes melatonin a more potent free radical scavenger than glutathione (Reiter et al., 2007b). The binding site of melatonin termed as MT_3 receptor is a detoxifying and antioxidant enzyme quinine reductase enzyme, the activation of which may also be responsible for some of the melatonin's anti-oxidative effects (Nosjean et al., 2000; Witt-Enderby et al., 2006; Tan et al., 2007).

1.4 Melatonin and Bone

Melatonin has been implicated in osteogenesis and osteolysis through a variety of mechanisms. Its osteogenesis or bone forming properties have been attributed to its effects on osteoblasts to either induce their differentiation (Roth et al., 1999; Radio et al., 2006) or proliferation (Nakade et al., 1999; Satomura et al., 2007), inhibit osteoclast activity (Koyama et al., 2002; Suzuki and Hattori, 2002) (Cardinali et al., 2003). A summary of various effects of melatonin on bone status is given below.

1.4.1 Human and Animal Studies

The prevalence of osteoporosis in women is 10 years following menopause, suggesting that circulating estrogen levels play a critical role in preventing bone loss in women. Besides estrogen, melatonin levels decrease with age and after menopause. These findings suggest that melatonin may be an important factor in the development of post-menopausal osteoporosis in women (Waldhauser et al., 1984; Sack et al., 1986). Bone metabolism has a diurnal rhythm which can be attributed to melatonin production in the body (Hassager et al., 1992; Greenspan et al., 1997; Heshmati et al., 1998; Ostrowska et al., 2003). Exposure to artificial light at night is related to decreases in plasma melatonin levels (Graham et al., 2001). In a study performed on women who worked night shifts for more than 20 years, there is a significantly increased risk of wrist and hip fractures compared with women who never worked at night (Feskanich et al., 2009). It is known that exposure to artificial light (as in the case of night shift workers) at night is related to decreases in plasma melatonin levels (Graham et al., 2001),. It could be assumed that increases in melatonin in the body can be protective against osteoporosis. Likewise, a negative correlation between the salivary melatonin levels and biochemical markers of bone resorption is found in post-menopausal obese women, again supporting that melatonin may be an endogenous inhibitor of osteoclast activity (Ostrowska et al., 2001a).

Several animal studies suggest that a relationship between melatonin levels and bone metabolism exists. Early studies demonstrate that pinealectomy affects the morphology of the parathyroid gland, a gland that controls calcium in the bone (Chen et al., 1990). Pinealectomy (removal of melatonin source) of chickens results in lower bone

mineral density in the vertebral column. These data suggest that endogeneous melatonin may have an osteoinductive effect on bone formation. Histological changes in pinealectomized chickens show that the total number of osteocytes is significantly lower than control, suggesting that melatonin enhances osteocyte proliferation in the cervical vertebra. (Turgut et al., 2005). High night-time levels of melatonin are found in bone marrow of rats and humans (Tan et al., 1999; Conti et al., 2000), and this could be a contributing factor in the differentiation of precursor cells to osteoblasts. In ovariectomized rats, there is an increase in bone resorption marker urinary deoxypyridinoline compared to non- ovariectomized rats. Ovariectomy is often used in experimental animals to mimic the conditions of post-menopausal osteoporosis. This effect is not observed in rats given an exogenous melatonin (25 mg/ml) dose in drinking water (Ladizesky et al., 2001). In another study on bilaterally ovariectomized rats, a decrease in trabecular thickness of the vertebra and a decrease in both trabecular and cortical thickness of the femur occurs that is prevented by exogenous melatonin (10 mg/kg) administration (Uslu et al., 2007). In male mice administered melatonin, a significant increase in bone mineral density and bone mass occurs compared to mice treated with vehicle alone (Koyama et al., 2002). These studies support the role of melatonin in bone remodeling processes.

Several studies reported a relationship between melatonin levels and scoliosis (a bone deformity characterized by curvature of spine). Removal of the pineal gland results in the development of scoliosis in chickens that has anatomical characteristics similar to those of human idiopathic scoliosis (Machida et al., 1993). In another study using C57BL/6J mice, it is shown that bipedal ambulation combined with melatonin deficiency

induces scoliosis. Ambulation in a mouse strain, which displayes normal melatonin rhythms, also results in scoliosis although at a rate slower than in C57BL/6J mice (Oyama et al., 2006). C57BL/6, a common strain of laboratory mice, is also a natural knock-down model for melatonin synthesis in the pineal gland, where N-acetyl transferase function is compromised because of a point mutation in its gene (Roseboom PH, 1998). From these studies, it is hypothesized that bipedal primates, like humans, may develop scoliosis when melatonin levels in the body are depressed (i.e., aged or light-at-night exposed individuals). However, this hypothesis is not supported in rhesus monkeys, where pinealectomy and ambulation do not cause scoliosis (Cheung et al., 2005). Nonetheless, in human adolescents with idiopathic scoliosis, serum melatonin levels are significantly lower compared to controls (Sadat-Ali et al., 2000). Also, osteoblasts from patients with adolescent idiopathic scoliosis show an impairment of melatonin- induced inhibition of forskolin stimulated adenylyl cyclase activity (Moreau et al., 2004). These data suggest that melatonin, acting through melatonin receptors and cAMP dependent pathway in osteoblasts, protect against scoliosis. Oral melatonin treatment in patients with adolescent idiopathic scoliosis and low melatonin levels delays the progression of this disease (Machida et al., 2009). These studies support a role for melatonin in preventing pathogenic bone conditions such as osteoporosis and scoliosis.

1.4.2 In- Vitro Studies

Various in-vitro studies have demonstrated the relationship between melatonin and bone. For example, murine and human bone marrow cells have been shown to have high concentrations of melatonin and serotonin-N-acetyltransferase activity, the rate limiting enzyme in melatonin synthesis (Conti et al., 2000). Melatonin significantly

increases the proliferation of HOB-M (normal human bone cells) cells and SV-HFO (human osteoblastic cell line) cells. Melatonin causes a dose-dependent reduction in both osteoclastogenesis and osteoclast activity to reduce the number of resorption pits and average pit area/pit in mouse bone marrow cells (Koyama et al., 2002). Inhibition of osteoclast activity, as measured via a reduction in tartrate-resistant acid phosphatase activity, is observed in cultures of goldfish scales (an in-vitro model for studying bone metabolism) (Suzuki and Hattori, 2002).

Mechanisms underlying melatonin-induced effects on osteoblast are not clear, and this may be dependent on the choice of the model system. For example, in human bone cells in culture, melatonin increases the expression of procollagen type I c-peptide, but is without effect on alkaline phosphatase and osteocalcin, the proteins involved in formation of bone (Nakade et al., 1999). However, in cultures of goldfish scales, melatonin decreases the expression of alkaline phosphatase and mRNA levels of estrogen receptor and of insulin-like growth factor- a growth factor involved in bone metabolsim (Suzuki and Hattori, 2002; Suzuki et al., 2008a). Positive effects on osteoblast differentiation are seen in pre-osteoblast and rat osteoblast-like osteosarcoma cell lines where treatment with 50 nM melatonin show an increase in bone marker proteins like alkaline phosphatase, osteopontin and osteocalcin. The effect of melatonin is inhibited by the melatonin receptor antagonist luzindole and by pertussis toxin, suggesting the involvement of melatonin transmembrane receptors (Roth et al., 1999). Melatonin increases the differentiation of human mesenchymal stem cells to osteoblasts, as measured by an increase in alkaline phosphatase activity (Radio et al., 2006). In a similar study, melatonin enhances the proliferation and differentiation of human osteoblasts, and

promotes the gene expression of type I collagen, osteopontin, bone sialoprotein, and osteocalcin (Satomura et al., 2007). Melatonin also stimulates osteogenic differentiation in bone marrow stem cells as measured by alkaline phosphatase activity and staining for calcium deposition (Zaminy et al., 2008). Thus, it can be seen that melatonin influences bone metabolism in-vitro, similar to its effects in-vivo.

1.5 Signaling Events Governing Osteoblast Differentiation

The differentiation of mesenchymal stem cells into osteoblasts is controlled by complex activities involving signal transduction pathways that are not fully understood. Mesenchymal stem cells are obtained from the bone marrow and are multipotent with the ability to differentiate into osteoblasts, chondrocytes and adipocytes (Pittenger et al., 1999). Stem cells are activated by tightly regulated and complex signals. Following initial lineage commitment by the stem cell, the cell divides giving rise to another stem cell (self-renewal) and a committed osteoprogenitor. The mature osteoprogenitor is able to divide to form pre-osteoblasts, which is an intermediate stage of differentiation. At this stage, the pre-osteoblasts begin to express certain bone markers like alkaline phosphatase (ALP), parathyroid hormone and type l collagen. The pre-osteoblasts undergo further differentiation to form mature osteoblasts, which express alkaline phosphatase, osteopontin, bone sialoprotein, and osteocalcin (Thomas and Kansara, 2006). The last stage of differentiation is the osteocyte. A range of cytokines modulate the differentiation processes, like BMP (bone morphogenetic proteins) and TGF-β (transforming growth factor-β) (Huang et al., 2007). These proteins are frequently used as markers for osteoblast differentiation, with the most common ones being alkaline phosphatase, osteocalcin, bone morphogenetic proteins, runt-related transcription factor -

2, and bone sialoprotein and type-1 collagen. Different stages of differentiation of human mesenchymal stem cells into osteoblasts are represented in Fig. 2.

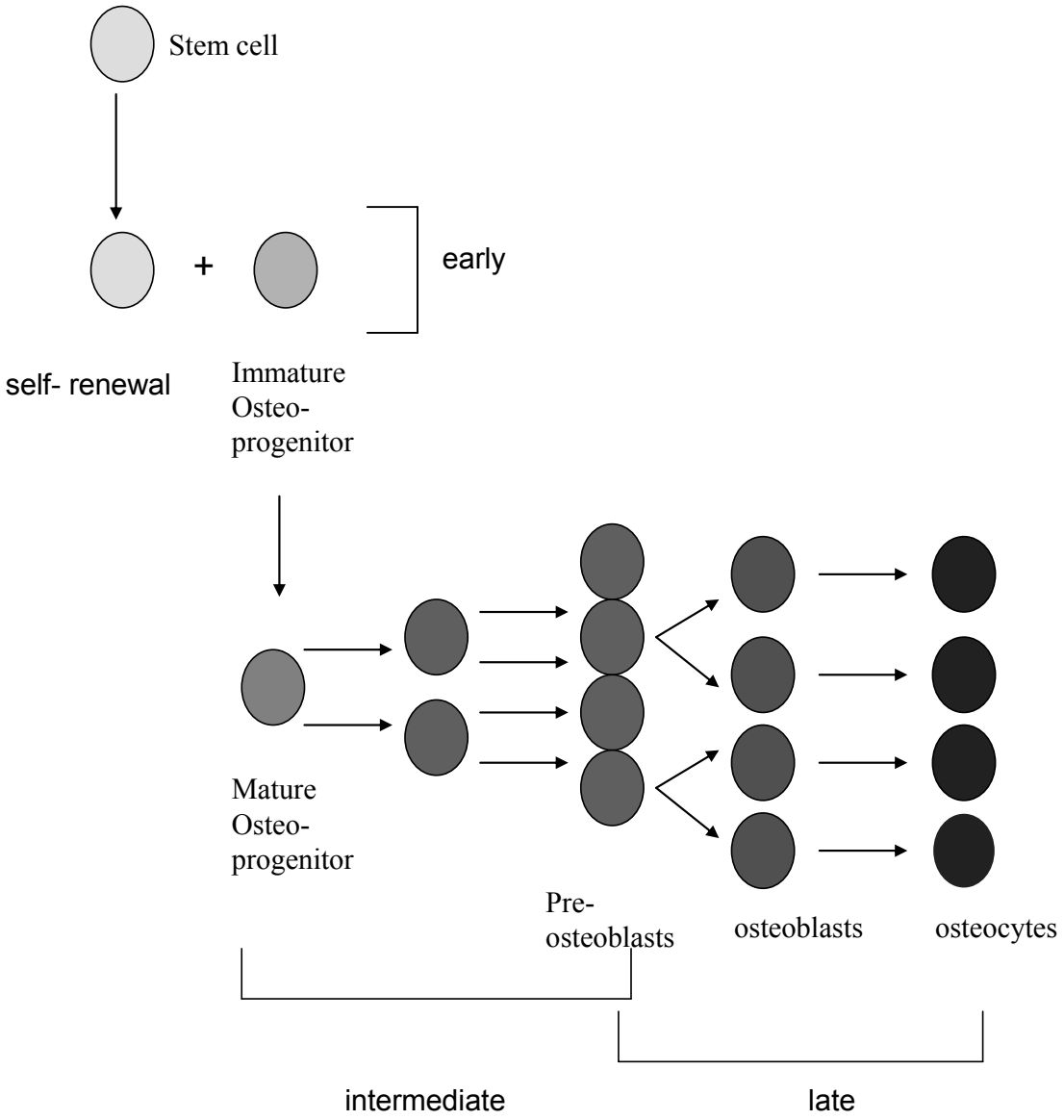


Figure 2: Diagrammatical Representation of the Different Stages of Differentiation of human Mesenchymal Stem cells into Osteocytes

Adapted from(Thomas and Kansara, 2006).

The most important transcriptional regulators of osteoblast differentiation are Runt-related transcription factor 2 (*RUNX-2*) and osterix (Official name: SP7). Knockout mice for these genes demonstrate a complete lack of ossification (Komori et al., 1997; Nakashima et al., 2002). *RUNX-2* regulates the transcription of other bone specific genes like osteocalcin and osteopontin (Ducy et al., 1999).

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily (Chen et al., 2004). The most readily detectable BMPs are BMP-2, -4 and -6 (Huang et al., 2007). BMPs bind to BMP receptors and signal downstream via Smad dependent or Smad independent pathways (Derynck and Zhang, 2003). Smads are the downstream signal transducers for the serine/threonine kinase receptors. Upon BMP stimulation and activation by TGFR receptors, receptor-regulated Smads (R-Smads) are phosphorylated, which in turn, form complexes with another type of Smad called common-partner Smads (Co-Smads). The R-Smad/ Co-Smad complexes then translocate into the nucleus where they interact with various transcription factors to regulate transcription of osteoblast specific genes (Chau et al., 2009). One of the transcription factors in the nucleus that is induced through this pathway is *RUNX-2* (Lee et al., 2003; Miyazono et al., 2005).

BMPs can also act through Smad independent pathways to activate MAPK (mitogen activated protein kinases) pathway and activate proteins ERK (Extracellular signal-regulated kinases) , JNK (c-Jun N-terminal kinases) and p38 in osteoblastic cells (Derynck and Zhang, 2003; Guicheux et al., 2003). ERK could activate *RUNX-2* downstream and increase its transcription activity (Ge et al., 2007). The MAPK pathway has been reported to be involved in differentiation of osteoblasts (Ahmed et al., 2003), as

measured by bone marker proteins alkaline phosphatase and osteocalcin (Ahmed et al., 2003; Guicheux et al., 2003; Radio et al., 2006).

Another signaling mechanism in osteoblast differentiation is the activation of Wnt-β-Catenin pathway. Wnts are released from the signaling cells and are ligands for FZD (membrane spanning frizzled receptor) (Huang et al., 2007). The activated receptor causes a downregulation of glycogen synthase kinase 3 and subsequent hypo-phosphorylation of its substrate β-Catenin. β- Catenin then translocates to the nucleus to activate osteoblastic gene expression of genes like *RUNX-2* and osteocalcin (Day et al., 2005). The various signaling pathways are depicted in Fig. 3.

In addition to the aforementioned pathways, various other signaling mechanisms i.e., fibroblast growth factors, Notch receptors, parathyroid hormone and molecules like Indian hedgehog are also involved in osteoblast differentiation (Huang et al., 2007; Chau et al., 2009). Adding to the complex mechanisms of bone differentiation is the fact that many of these pathways interact with each other. For example. the BMP-Smad pathway and the Wnt-β-Catenin pathway have been reported to interact in osteoblasts (Guo and Wang, 2009).

In the present study, various genes mentioned above were investigated to determine which of these pathways is influenced by melatonin.

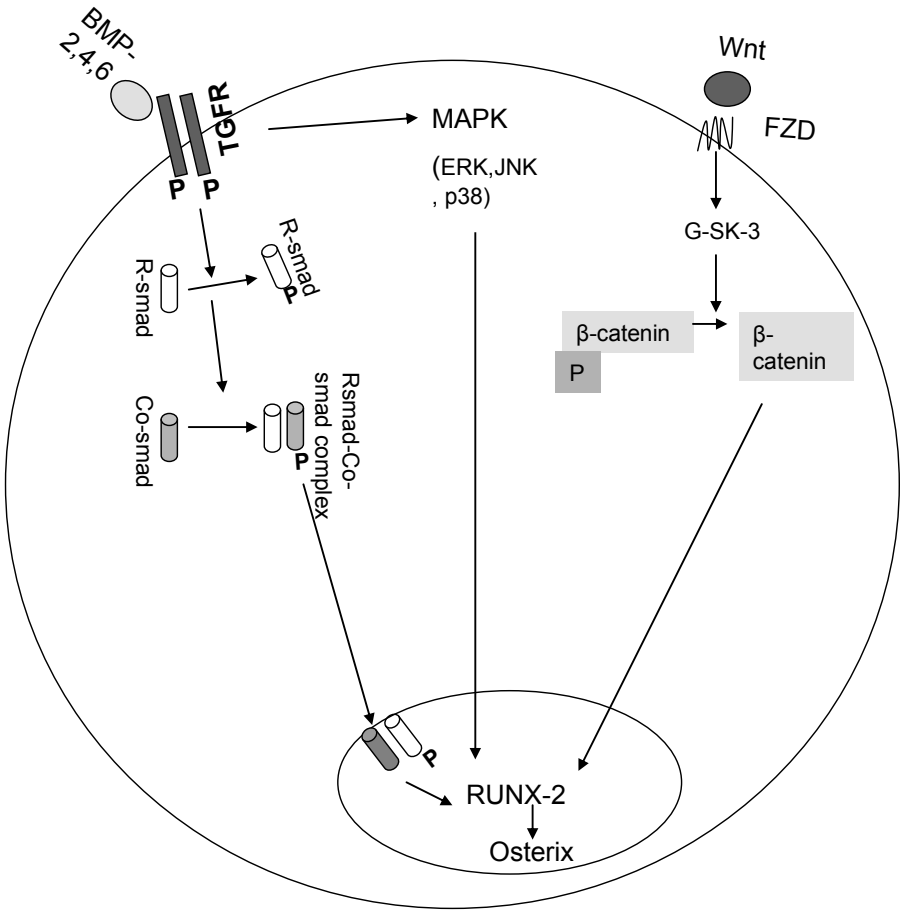


Figure 3: Important Signaling Mechanisms Involved in Osteoblast Differentiation

Bone morphogenetic proteins can increase the expression of *RUNX-2* and osterix through Smad dependent pathways or through activation of MAPK. *RUNX-2* may also be activated through Wnt binding to FZD receptors. *RUNX-2* and osterix expression is vital for osteogenesis.

1.6 Mechanisms of Action of Melatonin on Osteogenesis

Melatonin's actions on bone involve many different signal transduction mechanisms comprising osteoclasts, osteoblasts, and interaction with estrogen (Witt-Enderby et al., 2006).

RANKL-RANK-OPG pathway: Studies have shown that melatonin inhibits osteoclast formation and activity and thus prevents excessive bone resorption. One of the main mechanisms for this is the interaction of melatonin with the RANKL-RANK-OPG pathway. Osteoblasts and stromal stem cells express a protein called receptor activator of NF-κB ligand (RANKL), which binds to its receptor, RANK, on the surface of osteoclasts and their precursors. The RANK-RANKL interaction increases the development of osteoclasts and their activity. Osteoprotegerin (OPG) is secreted by osteoblasts and protects the skeleton from excessive bone resorption by acting as a decoy by binding to RANKL and preventing it from interacting with RANK (Wada et al., 2006; Boyce and Xing, 2007) (Fig. 4). In a study using mouse osteoblastic MC3T3-E1 cells, it was shown that melatonin (5-500 μM) decreases RANK mRNA and increases mRNA and protein levels of osteoprotegerin. This study demonstrates that melatonin increases bone mass and reduces bone resorption by down-regulating RANK-mediated osteoclast formation and activation (Koyama et al., 2002; Cardinali et al., 2003).

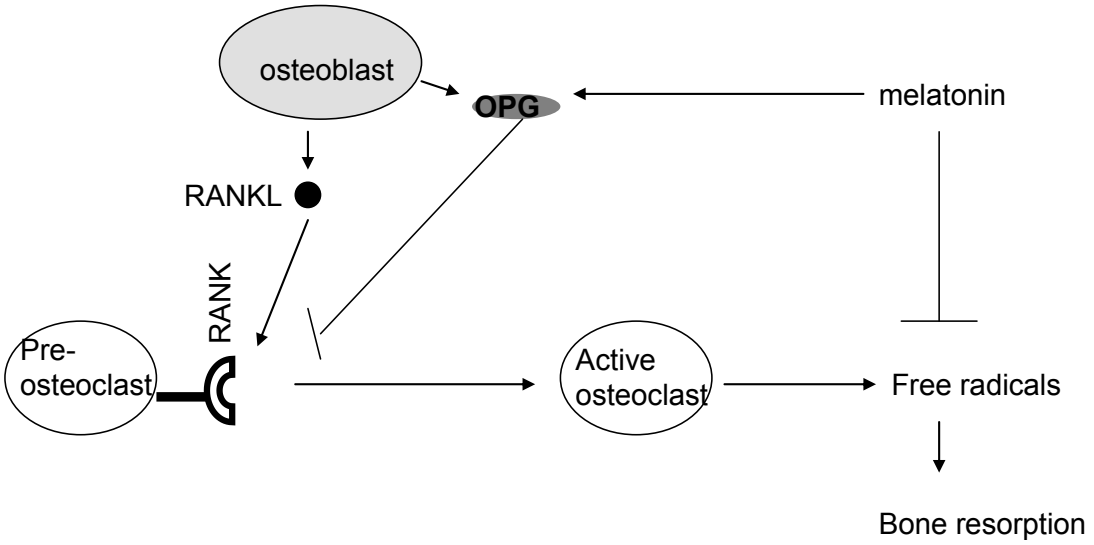


Figure 4: Schematic Representing Melatonin's Effects on Osteoclasts

Pre-osteoclasts express receptor RANK (receptor activator of NF-κB) on their surfaces. Activation of these receptors by protein RANKL, secreted by osteoblasts, lead to formation of active osteoclasts. OPG (osteoprotegerin), another protein secreted by osteoblasts acts as a decoy for RANKL and inhibit the formation of osteoclasts. Melatonin increases the expression of OPG, thus inhibiting osteoclastogenesis. Active osteoclasts secrete free radicals that can be scavenged by melatonin.

Anti-oxidant mechanisms of melatonin: Osteoclasts generated free radicals facilitate the destruction of calcified tissue, thus assisting in bone remodeling (Fraser et al., 1996; Berger et al., 1999; Yang et al., 2001). The accumulation of reactive oxygen species (ROS) is shown to increase gene transcription of RANKL and osteoclastogenesis by activation and nuclear accumulation of ERK, followed by phosphorylation of STAT3 protein (signal transducer and activator of transcription). This effect is reversed by the anti-oxidant *N*-acetyl cysteine (Chen et al., 2008b). In a case control study in healthy and osteoporotic individuals, oxidative stress was found to be an independent risk factor for osteoporosis measured through various oxidative markers (Sanchez-Rodriguez et al., 2007). These findings raise the possibility of preventing or treating bone loss by treatment with anti-oxidants (Banfi et al., 2008). As mentioned earlier, melatonin has free radical scavenging and anti-oxidant properties (Reiter et al., 2001; Reiter et al., 2002; Reiter et al., 2007a; Tengattini et al., 2008), thus, the inhibitory effect of melatonin on osteoclasts could partly be due to these effects (Fig. 4).

Involvement of melatonin receptors and MAPK pathway: Positive effects of melatonin on osteoblast differentiation have shown an involvement of the MT_2 receptors, activation of EGFR (epidermal growth factor receptor) and downstream activation of MAPK in human mesenchymal stem cells. It has been proposed that chronic activation of MT_2 receptors by melatonin results in MT_2R phosphorylation, and internalization by binding to β-arrestin. The activation of melatonin receptors by melatonin also leads to the activation of G_i proteins resulting in the dissociation of the G_i into its $G_{i\alpha}$ and $G_{\beta\gamma}$ subunit.

It is hypothesized that $G_{\beta\gamma}$ subunits activate matrix metalloproteinases within the membrane resulting in HB-EGF (heparin bound- epidermal growth factor) shedding to release EGF and activate the EGFR. β-arrestin is thought to form a scaffold with these proteins and ultimately cause MAPK (ERK1/2) activation. Activated MAPKs like p38, and ERK1/2 can promote the expression of differentiation markers of osteoblasts (Suzuki et al., 1999; Rawadi et al., 2001; Radio et al., 2006; Matsushita et al., 2009).

Involvement of estrogen receptor signaling: One of the putative causes of post-menopausal osteoporosis is a decline in estrogen levels. There is also a decline in melatonin levels with age and it is speculated that both these factors may combine to increase the risk of fractures in post menopausal women (Lindsay et al., 1976; Horsman et al., 1977; Waldhauser et al., 1984; Sack et al., 1986; Christiansen, 1993). Several investigators studying the anti-cancer properties of melatonin have demonstrated that melatonin has an inhibitory effect on estrogen receptor signaling mechanisms, as mentioned previously (Grant et al., 2009). Some studies have shown that melatonin is able to reverse the decrease of bone markers (trabecular thickness of vertebra and femur urinary deoxypyridinoline, a marker of bone resorption) induced by ovariectomy in animals (Ladizesky et al., 2001; Uslu et al., 2007). In a study by Suzuki and Hattori, it is shown that melatonin treatment depresses markers of osteoblastic differentiation including mRNA expression of estrogen receptor and insulin-like growth factor in scales of goldfish (Suzuki and Hattori, 2002). These findings are in contrast to most published studies examining the effect of melatonin on bone cell differentiation markers. The contradictory results of this study could be attributed to many reasons. For example, this

study was performed in scales of goldfish where ERβ seems to be largely related with osteoblastic activity and differentiation (Yoshikubo et al., 2005). The inhibitory actions of melatonin on ER has been reported mainly through ERα and not ERβ in MCF-7 cells (Martinez-Campa et al., 2006; Grant et al., 2009). Also, the study by Suzuki and Hattori reports an increase in osteoclast differentiation along with a decrease in osteoblast differentiation, an effect not seen in most other model systems. In light of most other published reports, both melatonin and estrogen have positive effects on bone formation, but exactly how each modulates the other's effect remains to be determined.

Epigenetic processes: Epigenetic processes contribute to development and differentiation of cells (Strathdee et al., 2004). Epigenetic gene regulation involves two main classes of molecular mechanisms: DNA methylation by DNA methyltransferases and histone modifications by histone deacetylases and histone acetyl transferases. DNA methyltransferases add methyl groups to the cytosines within CpG dinucleotides. The methylation of CpGs within genes serves to 'silence' their expression. Acetylation of histones by histone acetyl transferases activates gene transcription by moving apart histone proteins to allow for the general transcriptional apparatus (i.e., RNA polymerase II) to bind to promoter regions and initiate gene transcription. By contrast, deacetylation of histones by histone deactylases, keeps histones tightly bound, thus preventing access of RNA polymerase II and accessory proteins to bind to and activate gene transcription. DNA methylation patterns are reported to be essential for normal cell development and differentiation (Arney and Fisher, 2004). Nuclear factor-κB (NF-κB) (RANK), which is involved in melatonin's positive effect on bone formation as described previously, is

involved in activation of *CYP19* gene (Koyama et al., 2002). This gene, also called cytochrome P450 19, encodes for aromatase enzyme, which is responsible for a key step in the biosynthesis of estrogens (Nelson DR, 1993). It has been speculated that melatonin may cause methylation of *CYP19* gene or deacetylation of *CYP19* histone leading to gene silencing, thus demonstrating melatonin's involvement in epigenetic processes (Cai et al., 2007; Korkmaz et al., 2009). Melatonin may play a role in inhibiting osteoclasts and promoting osteoblast differentiation through epigenetic modifications of genes involved in bone development. A recent study has demonstrated that differentiation of neural stem cells by melatonin increases histone deacetylase mRNA levels and histone H3 acetylation (Sharma et al., 2008). Future work assessing the role of melatonin on epigenetic mechanisms may provide valuable mechanistic data underlying melatonin's effects on their differentiation into osteoblasts.

1.7 Human Adult Mesenchymal Stem Cell Model

The present study utilizes human mesenchymal stem cells as a model to study the effects of melatonin on osteoblast differentiation. Human mesenchymal stem cells are bone marrow-derived multipotent precursors capable of differentiating into chondrocytes, adipocytes, or osteoblast terminal cells (Pittenger et al., 1999). These cells are being studied for their potential in tissue regeneration (Huang and Li, 2008; Arthur et al., 2009; Karp and Leng Teo, 2009).

In the present study, human mesenchymal stem cells were cultured in osteogenic medium to drive their lineage to osteoblasts. Different time periods of melatonin exposure were studied to define the type of melatonin exposure required to drive highest osteoblast differentiation (section 3.1). Elucidating which type of melatonin exposure is

most effective will provide important clues as to the molecular mechanisms underlying melatonin's effects on bone metabolism, and also provide indications to determine the optimum method of administering melatonin to patients. Various osteogenic genes were also investigated in melatonin treated cells to determine the inter-cellular pathways involved in this process (section 3.10).

2. MATERIALS AND METHODS

2.1 Cell Culture

Multipotent adult human mesenchymal stem cells (hAMSCs) were were maintained at 37^0C and 5% CO_2. Cells were grown and passaged in mesenchymal stem basal cell medium containing 10% bovine growth serum (containing L-glutamine) (Lonza, Walkersville, MD). This medium was termed as OS- medium and was intended to promote hAMSCs proliferation, but not to induce differentiation into osteoblasts. For passing the cells, the cells were grown until they were 80% confluent in 75 cm^2 cell culture flasks, washed with 1 ml trypsin, then detached from the plate using 2 ml trypsin for 2-3 minutes. The cells were seeded using 3 ml trypsinate and 7 ml OS- medium. For various assays, the cells were seeded at an initial density of 3 X 10^3 cells/cm^2 in tissue culture plates, dishes or flasks.

For the studies assessing melatonin-dependent induction of osteogenesis, the cells were incubated in stem cell growth medium supplemented with 0.1 μM dexamethasone, 10 μM β-glycerphosphate and 50 μM ascorbate (OS+ medium) for the indicated times. This medium has been shown to enhance alkaline phosphatase (ALP) activity (a marker for

osteoblast differentiation) in several immature bone progenitor models (Cheng et al., 1994; Otsuka et al., 1999; Shiga et al., 2003; Jorgensen et al., 2004; Radio et al., 2006).

2.2 Treatment Groups

The treatment consisted of two study designs-Study Design A and Study Design B. The total time frame of treatment in both designs was 21 d. In the control group for both Study Designs A and B, the cells were incubated in OS+ for 21 d (days). In Study Design A, 50 nM melatonin was added to the medium before the end of the total period for the indicated times (2d, 5d, 10d, 14d and 21d). In Study Design B, 50 nM melatonin was added to the medium on day 1, the cells were treated with melatonin for the indicated times (2d, 5d, 10d, 14d and 21d), and then melatonin was removed and cells were cultured in control media without melatonin till a total period of 21 d. Media was removed and fresh media containing melatonin was replaced every 2 days. These two study designs were performed as such, so as to determine when melatonin exerts its maximal effect in stimulating osteogenesis- at a later stage of osteoblastic differentiation (Study Design A) or the initial phase of differentiation (Study Design B). These study designs and treatment groups would also indicate if short term or longer melatonin exposure was favorable to osteogenesis. The study designs are depicted diagrammatically in Fig. 5.

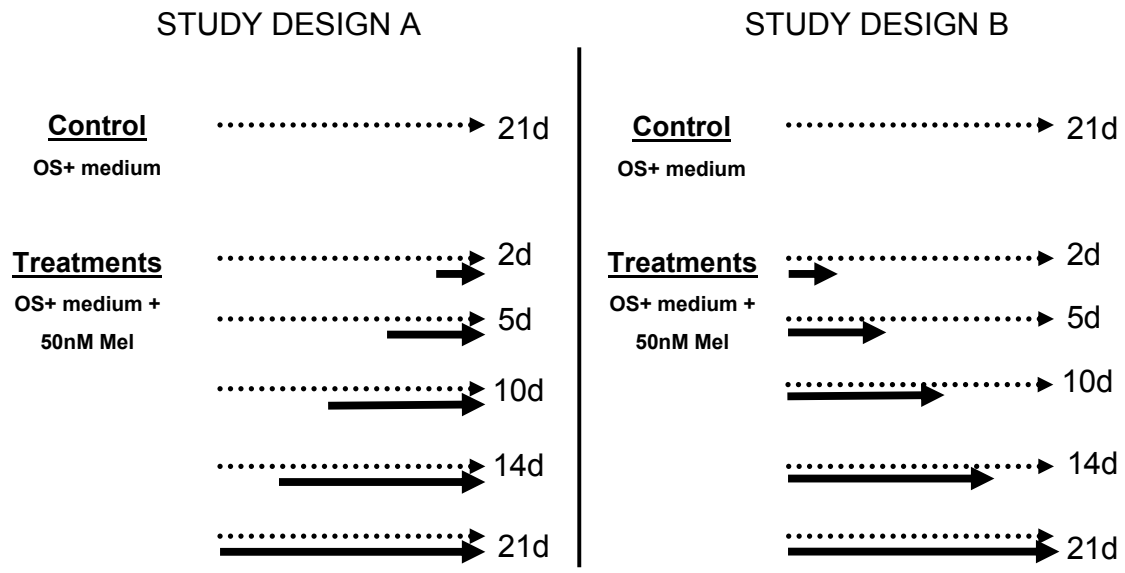


Figure 5: The Study Design

All treatments were carried out for 21 d. The hAMSCs in the control groups in both study designs were treated with OS+ medium for 21 d (broken lines). The treatment groups consisted of hAMSCs treated with OS+ medium (21 d) in addition to 50 nM melatonin (solid lines) for indicated times. In Study Design A, melatonin was added to the medium towards the end of the 21 d culture. In Study Design B, melatonin was added to the medium at the beginning of the 21 d culture. Media was removed and fresh media containing melatonin was replaced every 2 days.

For the antagonist studies, 1μM of MT_2 specific antagonist 1 μM 4P-PDOT (Tocris, Ballwin, MO) was added to the medium in addition to 50 nM melatonin for the indicated times.

For the experiment investigating the effect of varying the duration of osteogenic medium (OS+) prior to the 2 d melatonin exposure, the cells were first incubated in OS+ medium for 11d, 13d, 15d, 17d or 19d and then combined with melatonin for additional 2d towards the end of the OS+ exposure, as indicated in Fig. 6.

Figure 6: The Study Design for Investigating the Effect of Varying the Duration of OS+ Medium on Melatonin-Induced Osteoblast Differentiation

The hAMSCs were exposed to OS+ medium for varying times prior to the 2 d melatonin exposure. Dotted lines represent total exposure to OS+ medium and solid lines represent melatonin exposure. The cells were plated in OS- medium and the above treatments were started the following day. Melatonin was added for 2 d towards the end of the treatments with OS+ medium for indicated times.

2.3 Alkaline Phosphatase Assay

To assess the activity of osteoblasts, ALP assays were performed. The ALP activity was analyzed by measuring the rate of hydrolysis of p-nitrophenyl phosphate disodium hexahydrate (pNPP) by the hAMSCs following treatments. The hAMSCs were plated at a density of 3 X 10^3 cells/cm^2 in 12 well plates as described earlier and treated according to Study Design A or B for 21 d or as indicated. At the end of the time period, the cells were washed 3 times with 0.5 ml PBS to remove osteogenic medium and melatonin. After the washings, 0.5 ml of PBS was added to the wells; the cells were scraped into PBS, transferred to microcentrifuge tubes and then centrifuged for 10 min. The whole pellet was then reconstituted with 200 µl PBS. The pNPP product was generated using a p-nitrophenol phosphate stock (Sigma, St. Louis, MO) and analyzed according to the manufacturer's instructions. A yellow end product was obtained that was analyzed spectophotometrically at 405 nM using a microplate reader. The results were compared against a standard curve. ALP activity was normalized against total protein, determined via BSA assay (Pierce, Rockford, IL). A sample of calculations for determining ALP activity is given in Appendix 12.1.

2.4 Quantitative Analysis of Osteogenesis

To quanitify the extent of calcium deposition by osteoblasts, alizarin red stains were performed. Briefly, the hAMSCs were plated in 24 well plates as described earlier and exposed to melatonin as described in Fig. 5. Following the 21 d treatments, osteogensis quantitation was carried out using a commercial quantitation kit (Chemicon, Temecula, CA), where the intensity of alizarin red staining was measured using a microplate reader. A sample of calculations for quantitative analysis of osteogenesis is

depicted in Appendix 12.2. At the end of the treatment periods, the cells were washed and fixed in 70% ethanol for 15 min and then quantified by spectophotometry at 405 nM.

2.5 Qualitative Analysis of Osteogenesis

To assess the extent of osteoblast differentiation visually, alizarin red staining was performed on cells following the melatonin treatment for 21 d as described in fig 5. The hAMSCs were plated at a density of 3 X 10^3 cells/cm^2 or 0.75 X 10^3 cells/cm^2 in 10 cm^2 dishes. Following the 21d exposures, the cells were washed with 0.5ml PBS and fixed in 10% formalin for 10 min at room temperature. The cells were then stained with 2% alizarin red solution (Sigma Chemical Co., St. Louis, MO) for 10 minutes at room temperature. Prior to viewing the stained cells under a light microscope at 400X, the cells were washed 3 times with pure water.

2.6 Radioligand Binding Assays

To assess the melatonin receptor expression levels in differentiating hAMSCs, total binding analysis using the radioligand 2-[^{125}I]-iodomelatonin was performed. Briefly, the hAMSCs were plated in 10 cm^2 dishes and cultured for different times in medium according to the Study Design A or B. After the completion of the treatment, the cells were washed twice with 5 ml PBS, scraped in LIFT buffer (10 mM KPO_4, 1 mM EDTA at pH 7.4) and pelleted through centrifugation at 1500 rpm for 5 min. The cells were resuspended in 5 ml of 50mM Tris (pH 7.4) solution and incubated at room temperature for 1 hr with 500pM of 2-[^{125}I]-iodomelatonin in the absence (total binding) and presence (non-specific binding) of 1μM melatonin. Reactions were terminated by the addition of ice-cold Tris-HCl solution (50 mM) and rapid filtration over glass-fiber filters presoaked

in 0.5% polyethylenimine solution. Each filter was washed twice with 5 ml of ice cold TRIS (50 mM) buffer. Radioactivity was determined in a gamma counter and total specific binding of 2-[^{125}I]-iodomelatonin was calculated by subtracting non specific binding from total binding. Binding was converted to fmoles and then normalized per mg protein. Protein determinations were made by the method of Bradford using Bio-Rad protein assay reagents by comparing the values against a standard curve (Bio-Rad, Hercules, CA).

2.7 cAMP Accumulation Assays

To assess melatonin receptor function in differentiating hAMSCs, cAMP assays were performed using a direct cyclic AMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI). Briefly, the hAMSCs were plated in 12 well plates at a density of 3 X 10^3 cells/cm^2 and exposed to melatonin as described in Study Design A, except that cAMP measurements were made following 2, 10 or 21 d melatonin exposure and then compared to vehicle treated cells. After the treatment, the medium was aspirated, washed with 0.5 ml PBS and then exposed to serum free media containing either 30 μM rolipram alone (basal), 30 μM rolipram and 100 μM forskolin (maximal accumulation) or 30 μM rolipram, 100 μM forskolin and 10 nM melatonin. The cells were then incubated for 20 min in an incubator at 37^0 C. Cyclic AMP accumulation was measured according to manufacturer's instructions. To measure the decrease of cAMP with melatonin exposure, forskolin was added to the culture medium to artificially increase the production of cAMP. The decrease in cAMP accumulation by melatonin was measured as a function of forskolin response. Melatonin mediated inhibition of forskolin-induced cAMP

accumulation was measured in each set of cells and then expressed as a percentage of forskolin response within each group.

2.8 Cell proliferation Assays

To assess the extent of proliferation in differentiating hAMSCs, the cell proliferation assays were performed using CellTiter 96AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega, Madison, WI). Briefly, the hAMSCs were plated in 12-well plates at a density of 3 X 10^3 cells/cm^2 and treated according to Study Design A for the indicated times, except that measurements were made following 2, 10 or 21 d melatonin exposure and then compared to vehicle treated cells. After the treatment, the cells were washed twice with PBS, 200 µl of culture medium was added and cells were scraped using a cell scraper. One hundred microliters of this cell suspension was transferred to a 96 well plate and with 20 µL of the Cell Titer solution for 4 hr at 37^0 C. The solution was read by spectophotometry at 405 nm according to manufacturer's instructions.

2.9 RNA Extraction

To assess gene expression patterns of known osteoblast markers in differentiating hAMSCs, quantitative real time RT-PCR was performed. Briefly, the hAMSCs were plated in 10 cm^2 dishes and treated according to Study Design A for the indicated times. After the treatment, the cells were washed with PBS and scraped in 1 ml TRIZOL. Total RNA was isolated and purified using the RNeasy RNA Extraction Kit (Stratagene, La Jolla CA). The cell suspension was filtered using the manufacturer's pre-filter spin cups. Seventy percent ethanol was added to the filtrate and the solution was then filtered

through the filter spin cups to bind total RNA to the spin cup membranes. This RNA was further purified by washing with a low salt and high salt solution according to the manufacturer's instructions. RNA was quantified using a spectrophotometer at an optical density of 260 nm. Purity was assessed by calculating the ratio of the optical density at 260 nm to the optical density at 280 nm. Samples having a purity ratio less than 1.8 or greater than 2.1 were discarded and new RNA extracts were prepared.

2.10 RT-PCR Reactions

Total RNA (250 ng) was reverse transcribed into cDNA using ABgene Reverse-iT^{TM} 1st strand Synthesis kit (ABgene, Rochester NY). The cDNA were obtained using random decamers as per manufacturer's instructions. Random decamers were chosen over oligo(dT) primers, so that reverse transcription of the desired target region is not dependent on its distance from the polyA tail. A no "RT reaction" was made for each sample that contained RNA and all reagents except the transcriptase enzyme to ensure that DNA was not contaminating the RNA.

2.11 Real-Time RT-PCR

Real-time RT-PCR was performed using cDNA samples in the iCycler using iQ SYBR Green Supermix to quantify the levels of *mtTFA* (mitochondrial transcription factor A); *PGC-1α* (PPAR coactivator-1 α*); Pol γ* (DNA polymerase γ); *NRF-1* (nuclear respiratory factor 1), *PDK* (pyruvate dehydrogenase kinase), *PDH* (pyruvate dehydrogenase), *LDH* (lactate dehydrogenase), *RUNX-2* (runt related transcription factor-2), *OC* (osteocalcin), *BMP-2* (bone morphogenetic protein-2), *BMP-6* (bone morphogenetic protein-6), *ALP* (alkaline phosphatase) genes . Forward and reverse

primers were designed for each gene sequence using Primer3 and PrimerQuest (Rozen and Skaletsky, 2000). Primer specificity for the target gene was verified by aligning primer and gene sequences using NCBI nucleotide BLAST. All primers were designed to span at least one intron/ exon boundary to ensure amplification of mRNA sequences and greatly reducing replication of DNA sequences. Primer sequences were ordered from Integrated DNA Technologies (Coralville, IA). Lyophilized primers were resuspended using RNase/ DNase free water to a final concentration of 100 pmol/ µl. Thermocycling reactions were was carried at 95 °C for 3 min followed by 50 cycles at 95 °C for 30 s (denaturation) and 60 °C for 60 s (annealing). Each well contained a final volume of 25 µl. RT and no RT volumes were 0.5 µl in each reaction well. Forward and reverse primers were used at a final concentration of 200 nM each. A total of 12.5 µl of SYBR green mastermix was added to each reaction. A melt curve was performed after each PCR reaction to ensure the presence of a single gene product. *GAPDH* was used as the housekeeping gene. The ΔC_t values were calculated for each sample by subtracting the threshold cycle value (C_t) of GAPDH from the C_t value of gene of interest. Lower C_t and ΔC_t values indicate higher gene abundance. The fold change versus the control group was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A sample of calculations for fold changes in gene expression is depicted in Appendix 12.3. For genes involved in mitochondrial biogenesis and anaerobic metabolism, i.e., *mtTFA* (mitochondrial transcription factor A), *PGC-1α* (PPAR coactivator-1 α) and *Pol γ* (DNA polymerase γ), *NRF-1* (nuclear respiratory factor 1), *PDK* (pyruvate dehydrogenase kinase), *PDH* (pyruvate dehydrogenase) and *LDH* (lactate dehydrogenase), RT-PCR was

performed using the LightCycler TaqMan Master kit as described (Chen et al., 2008a). A list of all primers used in this analysis is shown in Table 1.

Name (human genes)	Entrez ID Number	Official Name	Forward Primer (5' 3')	Reverse Primer (3' 5')
GAPDH	NM_002046	GAPDH	GAGTCAACGGATTTGGTCGT	CATTGATGACAAGCTTCCCG3
MtTFA	NM_003201	TFAM	CAACTACCCATATTTAAAGCTCAGAA	GAATCAGGAAGTTCCCTCCA
PGC-1α	NM_013261	PPARGC1A	TGAGAGGGCCAAGCAAAG	ATAAATCACACGGCGCTCTT
Pol γ	NM_002693	POLG	CGTCTGACATACCACGTACCC	CACACGGCTGGTCATAAACTC
NRF-1	NM_005011	NRF1	TTGGAGAATGTGGTGCGTAAGT	GAGAGGCGGCAGTTCTGAGT
PDH	NM_000284	PDHA1	TCCGAGAGGCAACAAGGTT	AAGTCTGCAGCTCCATCAGG
PDK	NM_002610	PDK1	CAAGACCTCGTGTTGAGACCT	ACGTGATATGGGCAATCCAT

Name (human genes)	Entrez ID Number	Official Name	Forward Primer (5' 3')	Reverse Primer (3' 5')
LDH	NM_005566	LDHA	GCAGATTTGGCAGAGAGTATAATG	GACATCATCCTTTATTCCGTAAAGAC
RUNX-2	NM_001024630	RUNX2	TCACCTTGACCATAACCGTC	ATACTGGGATGAGGAATGCG
OC	NM_199173	BGLAP	TACCTGTATCAATGGCTGGG	ATGTGGTCAGCCAACTCGT
BMP-2	NM_001200	BMP2	AGAAGGAGGAGGCAAAGAAA	AAGCAGCAACGCTAGAAGAC
BMP-6	NM_001718	BMP6	CTAGCAATCTGTGGGTTGTGACTC	CCTCACTCACTTTGAAGAAAGCC
ALP	NM_000478	ALPL	ACACTGAAATATGCCCTGGA	GAAGGGGAACTTGTCCATCT

Table 1: Primers for Real-Time RT-PCR

Abbreviations: *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase); *mtTFA* (mitochondrial transcription factor A); *PGC-1α* (PPAR coactivator-1 α); *Pol γ* (DNA polymerase γ); *NRF-1* (nuclear respiratory factor 1), *PDK* (pyruvate dehydrogenase kinase), *PDH* (pyruvate dehydrogenase), *LDH* (lactate dehydrogenase), *RUNX-2* (runt related transcription factor-2), *OC* (osteocalcin), *BMP-2* (bone morphogenetic protein-2), *BMP-6* (bone morphogenetic protein-6), *ALP* (alkaline phosphatase).

2.12 Statistical Analysis

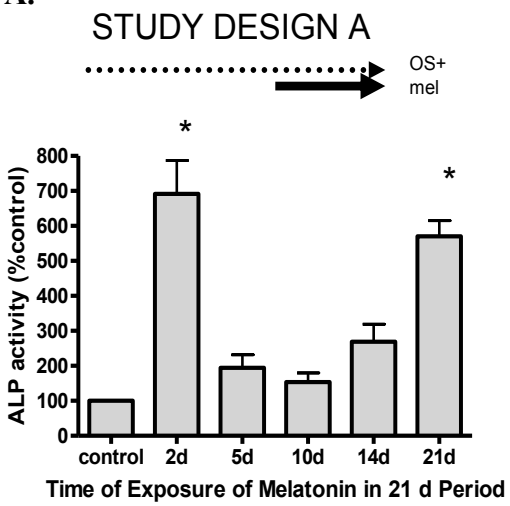
Statistical analysis was performed using commercial software (GraphPad PRISM®, GraphPad Prism, Inc., San Diego, CA), whereby a significant statistical difference was defined as $p < 0.05$. Data were analyzed by Student's t-test or one-way analysis of variance (ANOVA) followed by Newman-Keul's post hoc test to determine significant difference between all groups compared to each other. Although there is no set consensus in the scientific community on the correct choice of post hoc tests, Newman-Keul's post hoc test was chosen because this test is designed to have more power than other commonly used post hoc tests like the Tukey test. Given the nature of this study using stem cells (that at any given time, were present in different stages of differentiation) from human donors (who presented another variable in the study), a huge inter-and intra variation in samples was expected. It was our understanding that the additional power of the Newman-Keul's post hoc test would help to determine the significant difference between means.

3. RESULTS

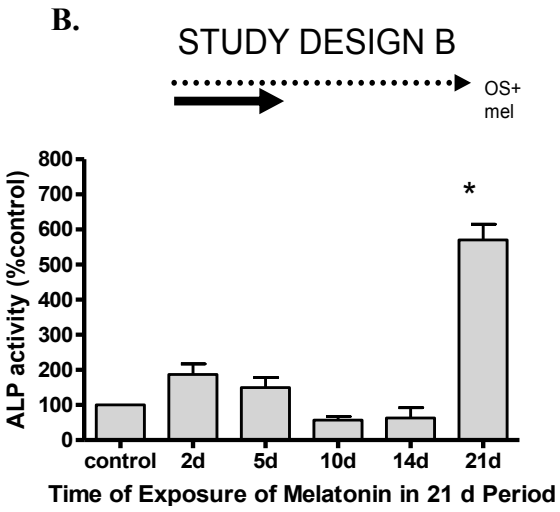
3.1 The Effect of Melatonin on ALP Activity in hAMSCs

To determine the treatment conditions that maximize melatonin-induced mesenchymal stem cell differentiation into osteoblasts, a marker for osteoblast activity, ALP was measured. As shown in Fig. 7, the addition of 50 nM melatonin to osteogenic medium was only with effect (increased ALP) if it remained in contact with the cells for the entire 21 d period or if it was added during the last 2 d of the 21 d treatment (Fig. 7A). There was no significant difference in ALP activity when the cells were exposed to 50 nM melatonin at the beginning of the OS+ 21 d treatment (stem cell growth medium supplemented with 0.1μM dexamethasone, 10 μM β-glycerphosphate and 50 μM ascorbate) (Fig. 7B). Because it was found that melatonin added during the last 2 d of the 21 d OS+ treatment induced ALP activity, the question was asked if time periods less than 2 d were sufficient for melatonin to induce ALP activity. As shown in Fig. 7C, no significant increase in ALP activity occurred in cells treated with melatonin for times less than 2 d compared to control cells. To determine whether effects of melatonin on inducing ALP activity was mediated through melatonin receptors, treatments were performed in the presence of 4P-PDOT, a selective MT_2 antagonist. As shown in Fig. 7D, the increase in melatonin-induced ALP activity at both 2 d and 21 d was blocked with the addition of antagonist 4P-PDOT to OS+ medium.

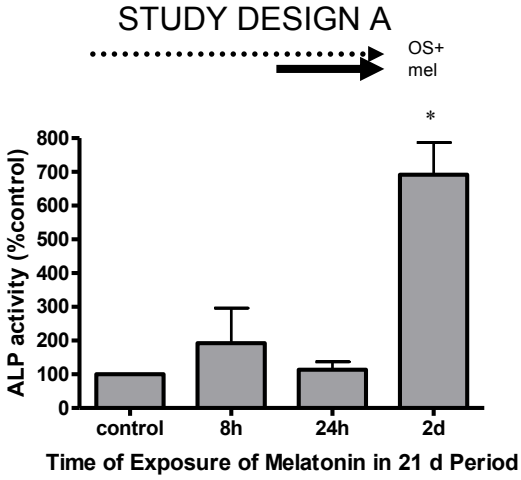
A.


STUDY DESIGN A
OS+
mel
*
*
800
700
600
500
400
300
200
100
0
ALP activity (%control)
control
2d
5d
10d
14d
21d
Time of Exposure of Melatonin in 21 d Period

B.


STUDY DESIGN B
OS+
mel
*
800
700
600
500
400
300
200
100
0
ALP activity (%control)
control
2d
5d
10d
14d
21d
Time of Exposure of Melatonin in 21 d Period

C.


STUDY DESIGN A
OS+
mel
*
800
700
600
500
400
300
200
100
0
ALP activity (%control)
control
8h
24h
2d
Time of Exposure of Melatonin in 21 d Period

D.

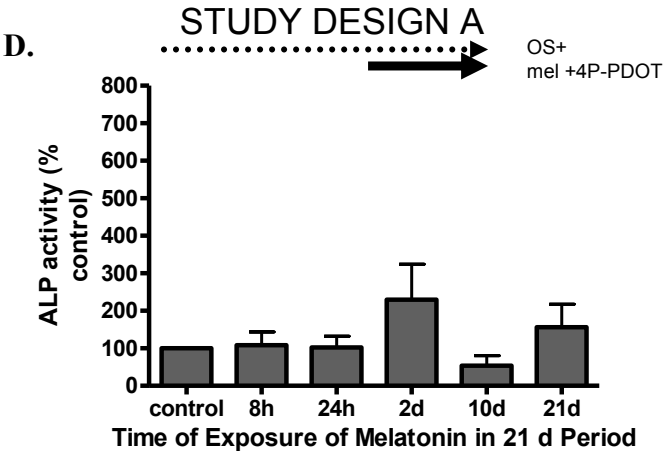

STUDY DESIGN A
OS+
mel +4P-PDOT
800
700
600
500
400
300
200
100
0
ALP activity (% control)
control
8h
24h
2d
10d
21d
Time of Exposure of Melatonin in 21 d Period

Figure 7: The Effect of Different Exposure Times on hAMSCs Differentiation into Osteoblasts

To assess the impact of different periods of exposure on melatonin-induced osteoblast differentiation, hAMSCs were plated at a density of 3 X 10^3 cells on a 12-well plate, and incubated with OS+ medium in the presence or absence of 50 nM melatonin. All cells were harvested on day 2 and the time points noted in the graph represent the length of treatments. For example, 2 d in graph A means cells began melatonin treatment on day 19 of OS+ treatment. Control treatment in all graphs represents cells grown in OS+ medium for 21 days. ALP activity increased when melatonin was added during the last 2 d of the 21 d treatment (A), or if it remained in continuous contact with OS+ medium for 21 d (A, B). Exposure to melatonin for less than 2 d was without effect on inducing ALP activity (C). These effects of melatonin were mediated through MT_2 melatonin receptors because the addition of 1μM 4P-PDOT (an MT_2 melatonin receptor antagonist) to the treatments containing both melatonin and OS+ medium blocked melatonin-induced increases in ALP activity (D). Each bar represents mean ± SEM of 3-5 experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t-test where significance (*) was defined as $p<0.05$ compared to vehicle (OS+) treated cells for 21 d.

3.2 ALP Levels in hAMSCs Exposed to OS+ medium over Different Periods of Time

To determine if pretreatment of hAMSCs with OS+ medium played a priming role in increasing ALP activity in cells treated with 50 nM melatonin for 2 d, hAMSCs were exposed to OS+ medium for varying lengths of time, before the melatonin was added for 2 d. As depicted in Fig. 8, 2 d melatonin exposure was still able to significantly increase ALP activity when hAMSCs were pre-exposed to OS+ medium for 17 d, however, the extent of ALP induction was less than hAMSCs pre-exposed to OS+ medium for 21 d.

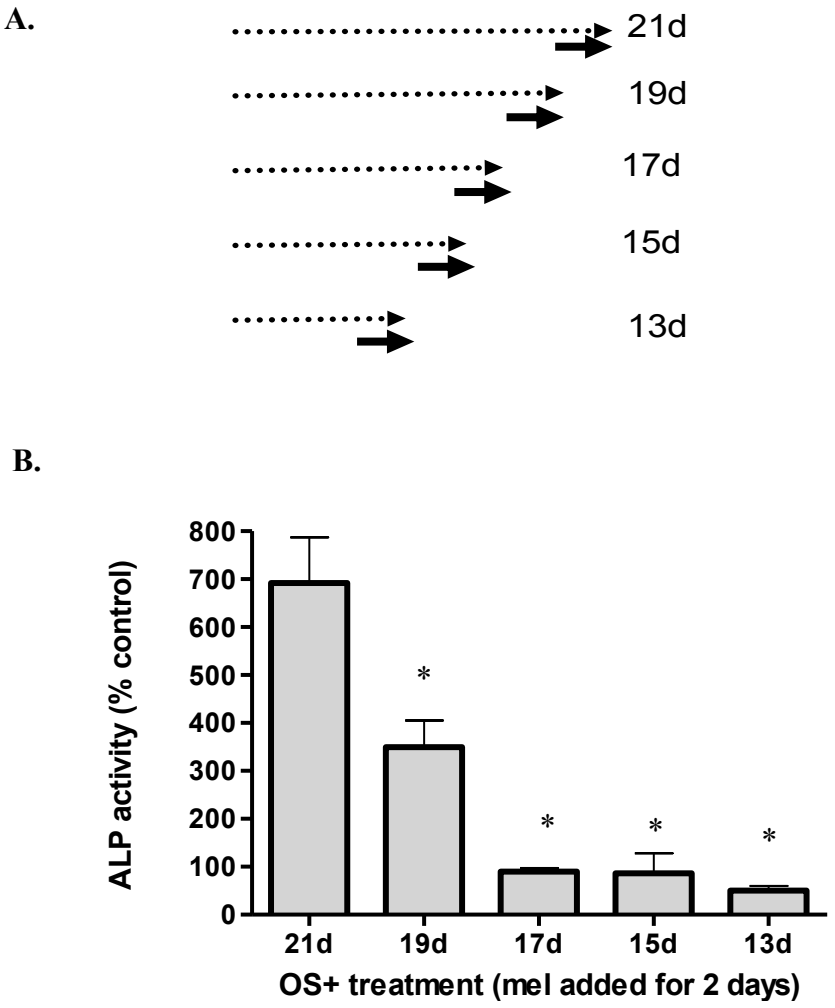


Figure 8: The Effect of Varying Pre-exposure to Osteogenic (OS+) Medium on 2 d Melatonin Induction of ALP in Differentiated hAMSCS

To assess the effect of pretreatment or priming of hAMSCs with OS+ medium on ALP activity, hAMSCs were plated at a density of 3 X 10^3 cells on a 12 well plate and exposed to OS+ medium for varying times prior to the 2 d melatonin (50 nM) exposure. Dotted lines represent total exposure to OS+ medium and solid lines represent melatonin exposure (A). ALP activity is represented as a percentage of activity in control cells (cells treated with OS+ medium in absence of melatonin). There was a significant decrease in ALP activity in hAMSCs exposed to OS+ medium for less than 21 d (B). Each bar represents mean ± SEM of 3 experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t-test where significance (*) was defined as $p<0.05$ compared to 21 d treatment.

3.3 Saturation Binding Analysis on hAMSCs Exposed to Melatonin over Different Time Periods

To determine if melatonin exposure affected the expression of melatonin binding sites in hAMSCs, saturation binding analysis was performed using 2-[^{125}I]-iodomelatonin on cells exposed to 50 nM melatonin for indicated times in Study Design A and Study Design B (Fig. 9). Other than the 10 d melatonin exposure in Study Design A, where there was an increase in the 2-[^{125}I]-iodomelatonin binding sites (3.86 fmol/mg protein) as compared to control (0.17 fmol/mg protein), there was no significant difference in the binding sites in any other treatment group vs. control, in both Study Design A and Study Design B (Fig. 9). The relative expression of 2-[^{125}I]-iodomelatonin binding in differentiating hAMSCs was between 0.17-1.2 fmol/mg protein.

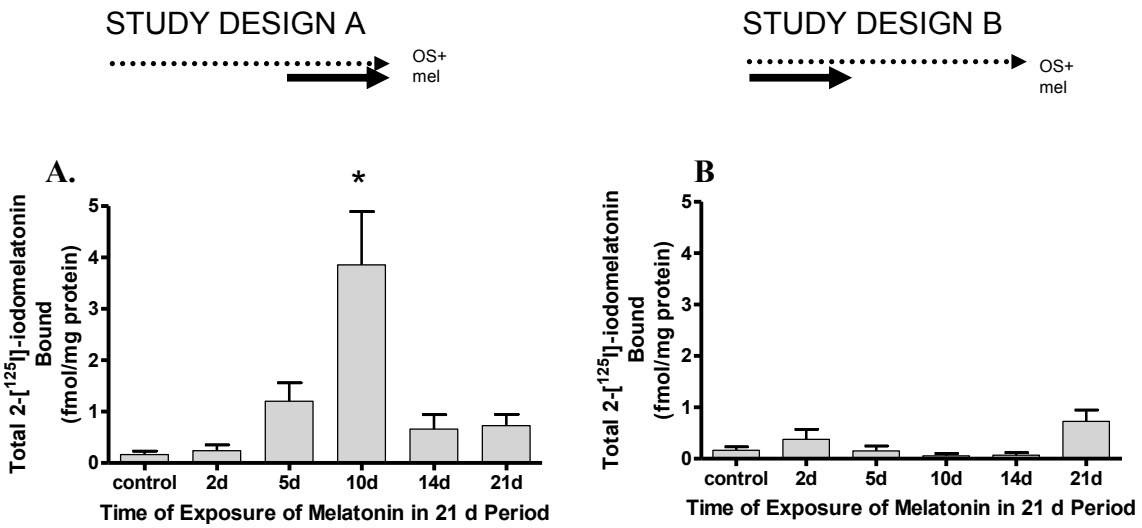


Figure 9: Total 2-[^{125}I]-iodomelatonin Binding Analysis on hAMSCs Treated with Melatonin over Different Time Periods

To assess whether or not melatonin receptor expression fluctuated in response to different treatment conditions, total melatonin receptor expression was measured using 2-[^{125}I]-iodomelatonin. hAMSCs were plated in 10 cm^2 dishes and exposed to melatonin for different times according to the Study Design A or B. 2-[^{125}I]-iodomelatonin binding sites were not found to be significantly different than control in both groups, except for 10 d melatonin exposure in Study Design A, where there was a significant increase in the 2-[^{125}I]-iodomelatonin binding sites. Each bar represents mean ± SEM of 7-20 experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test where significance (*) was defined as $p<0.05$, when compared to vehicle-treated cells.

3.4 Functional Analysis of Melatonin Receptors Expressed in Differentiating hAMSCs Exposed to Melatonin over Different Time Periods

To access the function of melatonin receptors, cAMP assays were performed on hAMSCs exposed to 50 nM melatonin for different times in Study Design A. Both MT_1 and MT_2 receptors are coupled mainly to G_i proteins that upon activation cause an inhibition of adenylyl cyclase enzyme in the cells (Grant et al., 2009). This leads to a decrease in second messenger cyclic AMP (cAMP) levels, PKA levels and CREB phosphorylation (McNulty et al., 1994; Witt-Enderby et al., 2003). To measure the decrease of cAMP with melatonin exposure, forskolin was added to the culture medium to artificially increase the production of cAMP. The decrease in cAMP accumulation by melatonin was measured as a function of forskolin response. hAMSCs in the control group (not treated with melatonin) showed a decrease in forskolin induced cAMP accumulation, suggesting sensitive melatonin receptors. Melatonin exposure for the last 2 d of a 21 d OS+ exposure resulted in melatonin receptor desensitization reflected by attenuation in melatonin-mediated inhibition of forskolin-induced cAMP accumulation (Fig. 10).

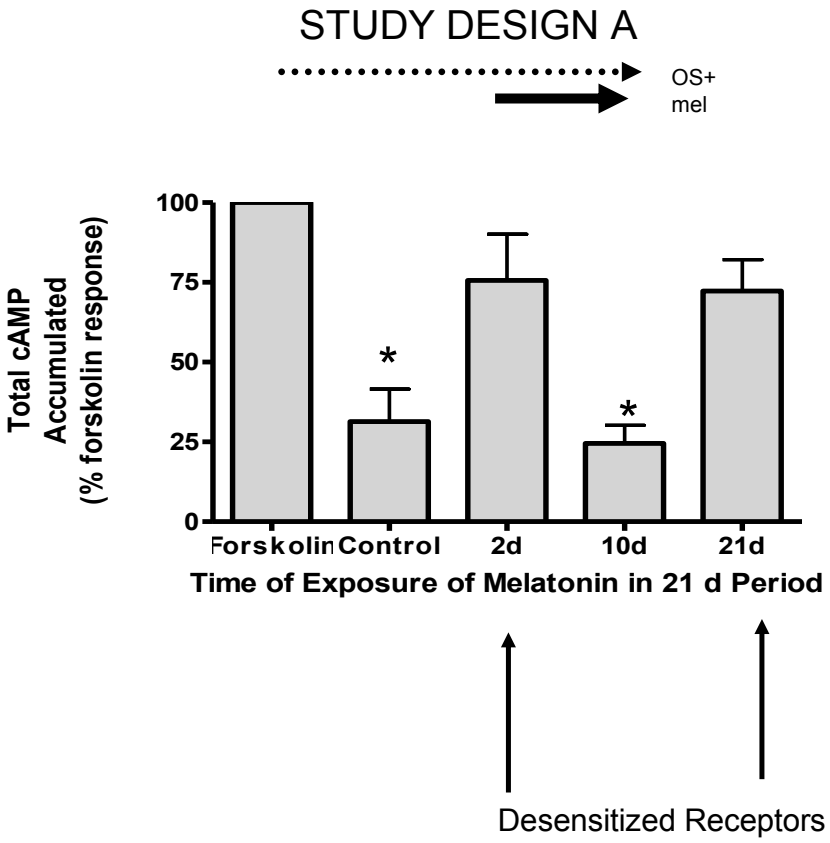


Figure 10: Functional Analysis of Melatonin Receptors Expressed in hAMSCs Exposed to Melatonin over Different Time Periods

To assess whether changes in melatonin receptor sensitivity occurred in response to differential exposure to melatonin, cAMP accumulation assays were performed. hAMSCs were plated in 12-well plates at a density of 3 X 10^3 cells/cm^2 and exposed to melatonin for 2, 10 or 21 d as described in Study Design A. Following exposure, hAMSCs were assayed for total cAMP accumulation using commercial cAMP kits. As shown, in vehicle-treated cells, 10 nM melatonin (a concentration known to decrease cAMP accumulation) inhibited forskolin induced cAMP formation. Similarly, melatonin inhibited cAMP formation in hAMSCs exposed to melatonin for 10 d according to Study Design A. There was no significant inhibition of forskolin induced cAMP accumulation when hAMSCs were exposed to melatonin for 2 d or 21 d in Study Design A. Each bar represents mean ± SEM of 3-5 experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test where significance (*) was defined as $p<0.05$, when compared to forskolin induced cAMP accumulation.

3.5 The Effect of Melatonin Exposure on Calcium Deposition in Differentiating hAMSCs

To determine the effect of differential melatonin exposure on calcium deposition (a marker of bone mineralization) in hAMSCs, calcium deposition assays were carried out using alizarin red staining in cells exposed to 50 nM melatonin. As shown, the deposition of calcium was increased by about 900% only in hAMSCs exposed to melatonin for entire 21 d (Fig. 11A). This melatonin mediated increase in calcium deposition was blocked in the presence of MT_2 melatonin receptor antagonist 4P-PDOT (Fig. 11B).

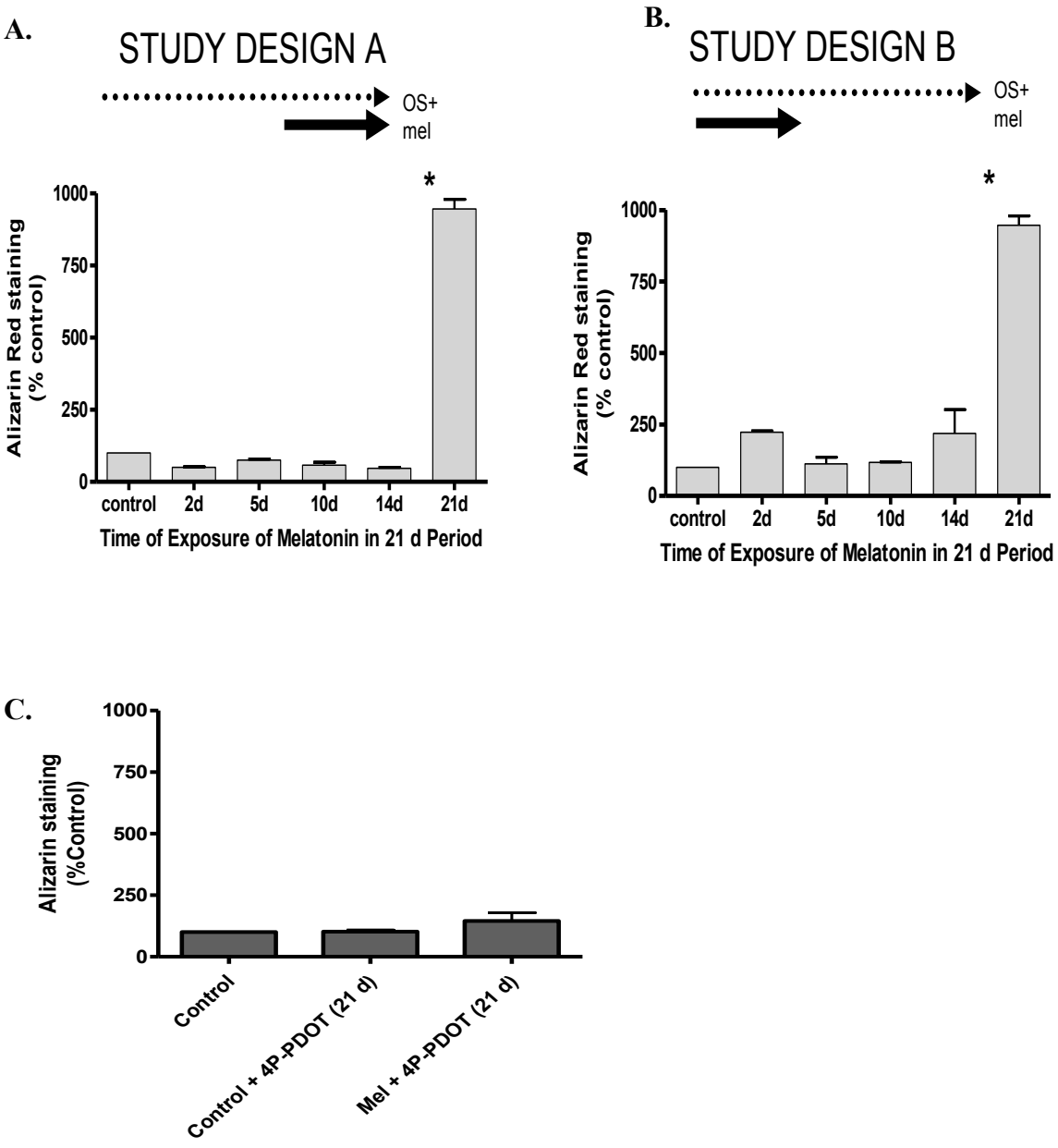


Figure 11: The Effect of Melatonin Exposures on Calcium Deposition in Differentiating hAMSCs

To assess whether or not exposure to melatonin-induced calcium deposition in differentiating hAMSCs, alizarin red staining assays were performed. Briefly, the hAMSCs were plated in 24 well plates at a density of 3 X 10^3 cells/cm^2 and exposed to OS+ medium containing melatonin. Following the various melatonin exposures in the 21d period, osteogenesis quantitation was carried out using commercial kits, in which the intensity of alizarin red staining was quantified. As shown, exposure to melatonin for 21 d induced calcium deposition by 900% compared to control (vehicle-treated cells) (A). No increases in calcium deposition occurred at times of melatonin exposure less than 21

d in Study Designs A and B (A and B). The melatonin-mediated increase in calcium deposition with 21 d exposure was blocked in the presence of MT_2 antagonist 4P-PDOT (1 μM) suggesting that these effects were occurring through MT_2 melatonin receptors. Each bar represents mean ± SEM of 3 experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test where significance (*) was defined as $p<0.05$ compared to vehicle-treated cells.

3.6 Qualitative Assessment of Calcium Deposition in Differentiating hAMSCs Exposed to Melatonin for 21 d

To qualitatively assess calcium deposition in hAMSCs exposed to melatonin for 21 d in presence of OS+ medium alizarin red staining was carried out. This was done to visualize the calcium deposition under the microscope and determine if few or all cells stain for calcium. The melatonin-treated cells depicted a higher level of staining compared to the control treated cells. The staining was uniformly distributed among the cells, demonstrating that melatonin-induced increase in calcium deposition was not localized to few cells, depicting that mineralization was uniform and not a result of a few cells laying down calcium in the matrix (Fig. 12).

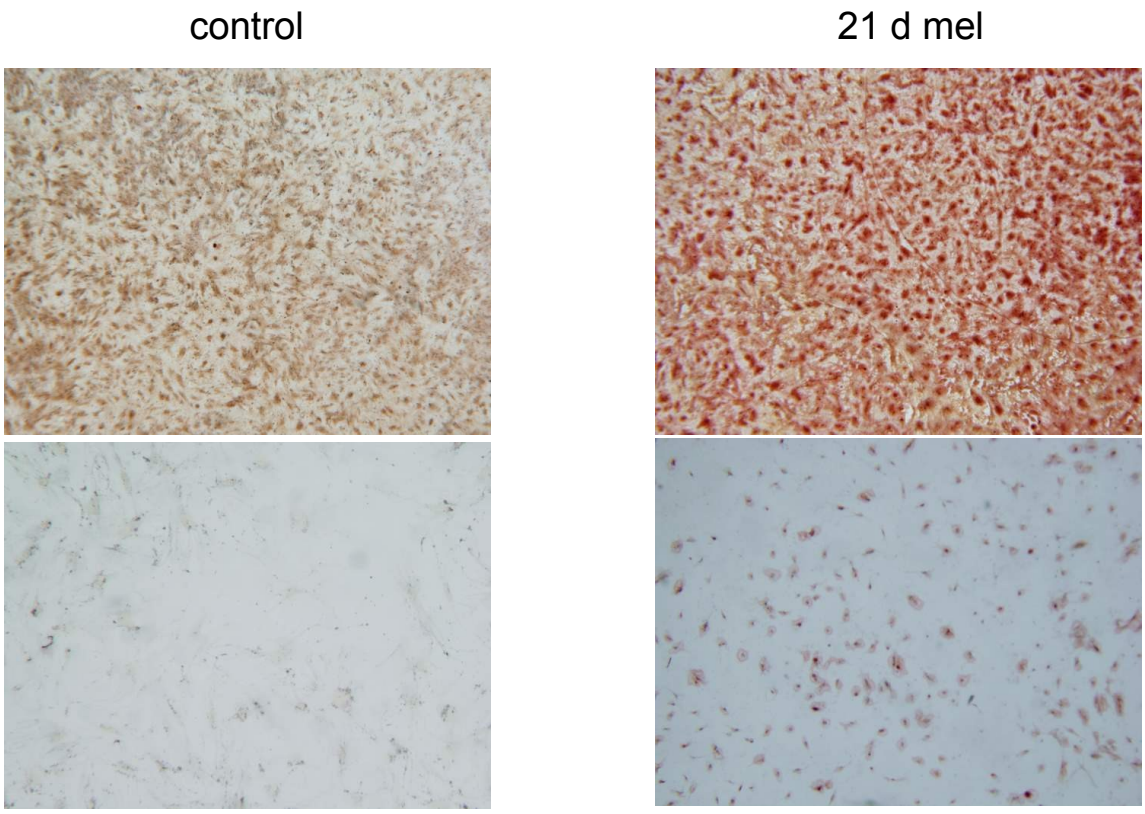


Figure 12: Qualitative Assessment of Calcium Deposition in Differentiating hAMSCs Exposed to Melatonin using Alizarin Red Staining

To assess the extent of calcium deposition by osteoblasts following a 21 d melatonin or OS+ exposure, alizarin red staining analysis was performed. Briefly, the hAMSCs were plated at a density of 3 X 10^3 cells/cm^2 (upper panel) or 0.75 X 10^3 cells/cm^2 in 10 cm^2 dishes (lower panel) in 10cm^2 dishes and exposed to OS+ medium in the absence or presence of 50 nM melatonin for 21 d. After treatments, the cells were washed and stained with 2% alizarin red solution. The red color indicates calcium deposition. As shown in pictomicrographs of hAMSCs after staining, there was a marked increase in staining in most of hAMSCs treated with melatonin compared to control treated cells. (Magnification= 400 X)

3.7 Proliferation of hAMSCs Treated with Melatonin for Different Times

To determine the effect of melatonin treatment on proliferation of hAMSCs, cell proliferation assays were carried out on cells treated with 50 nM melatonin for the indicated times according to Study Design A, as described in Materials and Methods. No change in proliferation of hAMSCs occurred in presence of melatonin compared to vehicle-treated cells (Fig. 13). The aim of this experiment was to determine if the changes in melatonin induced changes in the cells were due to changes in their proliferation.

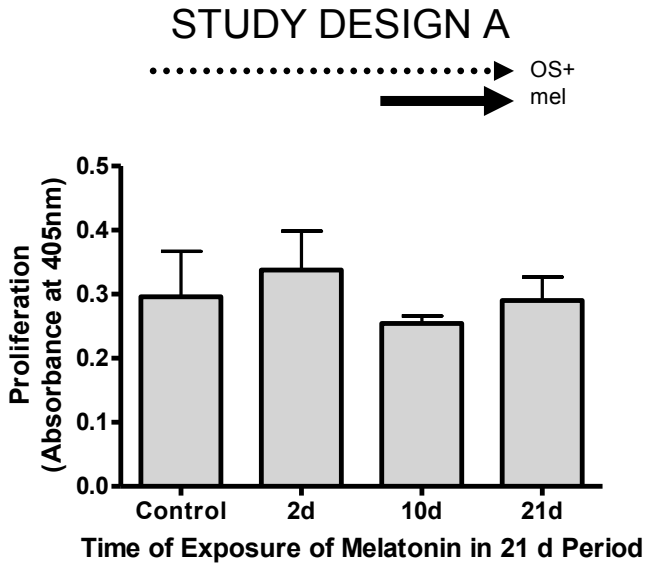


Figure 13: Proliferation of hAMSCs Treated with Melatonin for Different Times

To assess whether melatonin exposures for 2, 10 or 21 d using Study Design A described in Materials and Methods, affected the proliferation of the differentiating hAMSCs, proliferation assays were conducted. Briefly, hAMSCs were plated in 12-well plates at a density of 3 X 10^3 cells/cm^2 and exposed to melatonin for 2, 10 or 21 d in presence of OS+ medium. The cell proliferation was determined using commercial CellTiter $96AQ_{ueous}$ One Solution Cell Proliferation Assay Kit . As shown, there was no significant difference in proliferation of cells treated with 50 nM melatonin for indicated times compared with the control treated cells. Each bar represents mean ± SEM of 3 experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t-test where significance (*) was defined as $p<0.05$, compared to vehicle-treated cells.

3.8 The Effect of a 21 d Melatonin Exposure on Mitochondrial Gene Expression in Differentiating hAMSCs

Many studies have reported on melatonin's action on mitochondria and its functions (Reiter et al., 2001). Studies have shown that mitochondrial function is related to the differentiation process (Duguez et al., 2002; Cho et al., 2006; Chen et al., 2008a). To assess whether or not changes in mitochondrial biogenesis occurred as a result of the 21 d melatonin exposure, real time RT-PCR studies were carried out. Specifically, the effect of melatonin on the mitochondrial genes, mitochondrial transcription factor A (*mtTFA*), PPAR coactivator-1 α (*PGC-1α*), DNA polymerase γ (*Pol γ*) and nuclear respiratory factor 1 (*NRF-1*) were examined. No difference was found in the expression of these genes in cells treated with 50 nM melatonin for 21 d compared to the vehicle-treated cells (Fig. 14).

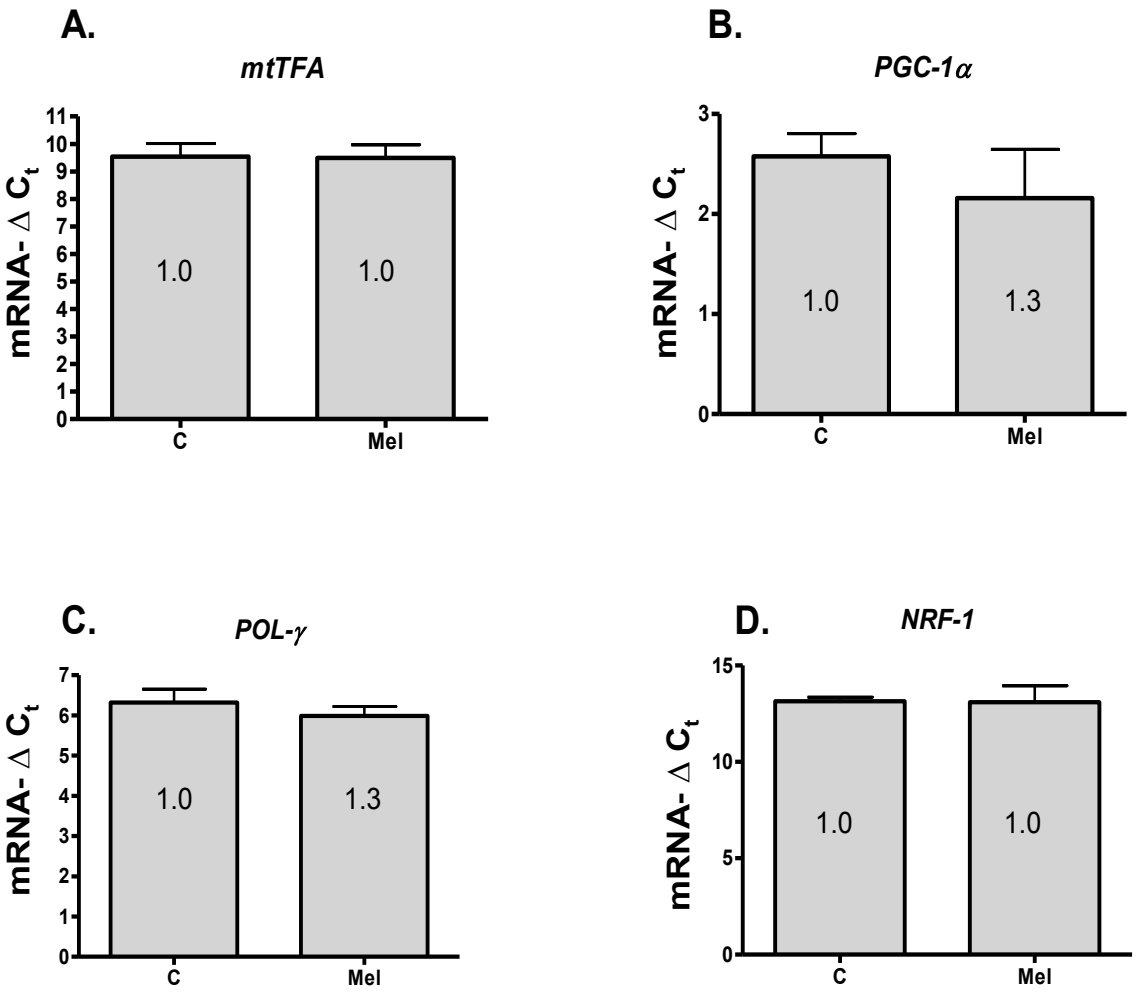


Figure 14: The Effect of a 21 d Melatonin Exposure on Mitochondrial Gene Expression in Differentiating hAMSCs

To assess whether or not changes in mitochondrial biogenesis within differentiating hAMSCs correlated with melatonin-mediated osteoblast differentiation, real time RT-PCR analysis was carried out. The cells were cultured in $10cm^2$ dishes in OS+ medium alone (control) or in presence of 50 nM melatonin (Mel) for 21 d. As described in Materials and Methods, cells were lifted in TRIZOL, total RNA was isolated and reverse transcribed into cDNA. Real-time RT-PCR analysis was carried out using primers specific for *mtTFA*, *PGC-1α* and *Pol γ NRF-1*. The primer sequences are given in Table 1. As shown, there was no significant difference in mRNA levels of mitochondrial biogenesis genes in hAMSCs treated with 50 nM melatonin for 21 d compared to vehicle-treated cells. ΔC_t values were determined by normalization to *GAPDH* to control for variation in cDNA levels between samples. Lower ΔC_t values indicate higher gene abundance. The relative fold changes over control are denoted as numbers in the bars. Each bar represents mean ± SEM of 4 experiments performed in duplicate. The data was analyzed by Student's t-test where (*) was defined as $p<0.05$, when compared to control. Abbreviations: *mtTFA* (mitochondrial transcription factor A), *PGC-1α* (PPAR coactivator-1 α) and *Pol γ* (DNA polymerase γ) and *NRF-1* (nuclear respiratory factor).

3.9 The Effect of a 21 d Melatonin Exposure on Genes Involved in Anaerobic Metabolism

It has been suggested in a previous study that there is a "metabolic shift" which reprograms the bioenergetic properties of hAMSCs from anaerobic glycolysis to mitochondrial respiration to meet the higher energy demand of differentiated osteoblasts (Chen et al., 2008a). To assess whether or not changes in anaerobic metabolism occurs as a result of the 21 d melatonin exposure, real time RT-PCR studies were carried out. Specifically, the effect of melatonin on genes involved in anaerobic metabolism, pyruvate dehydrogenase kinase (*PDK)*, pyruvate dehydrogenase (*PDH*) and lactate dehydrogenase (*LDH*) were examined. No difference was found in the expression of these genes in cells treated with 50 nM melatonin for 21 d compared to the vehicle-treated cells (Fig. 15).

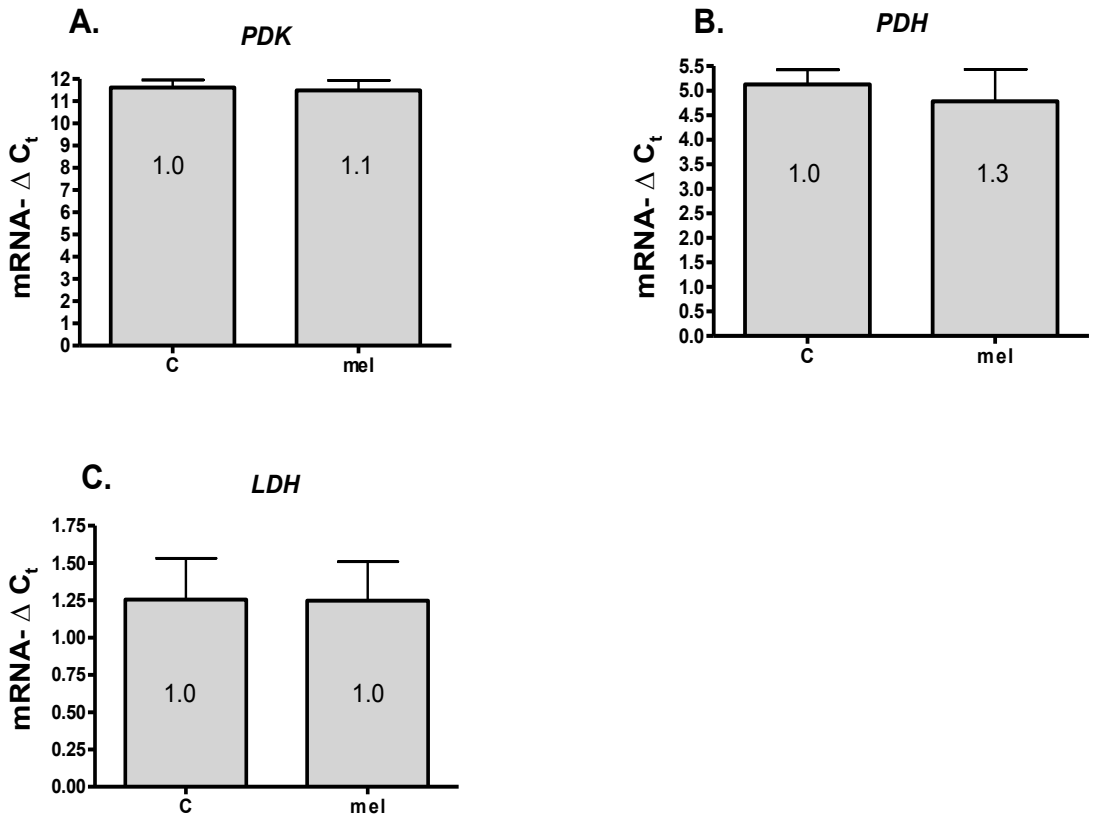


Figure 15: The Effect of a 21 d Melatonin Exposure on Genes Involved in Anaerobic Metabolism

To assess whether or not changes in anaerobic metabolism within differentiating hAMSCs correlated with melatonin-mediated osteoblast differentiation, real time RT-PCR analysis was carried out. The cells were cultured in 10cm^2 dishes in OS+ medium alone (control) or in presence of 50 nM melatonin (mel) for 21 d. As described in Materials and Methods, cells were lifted in TRIZOL, total RNA was isolated and reverse transcribed into cDNA. Real-time RT-PCR analysis was carried out using primers specific for *PDK*, *PDH* and *LDH*. The primer sequences are given in Table 1. As shown, there was no significant difference in mRNA levels of genes involved in anaerobic metabolism in hAMSCs treated with 50 nM melatonin for 21d compared to control treated cells. ΔC_t values were determined by normalization to *GAPDH* to control for variation in cDNA levels between samples. Lower ΔC_t values indicate higher gene abundance. The relative fold changes over control are denoted as numbers in the bars. Each bar represents mean ± SEM of 4 experiments performed in duplicate. The data was analyzed by Student's t-test where (*) was defined as $p<0.05$, when compared to control. Abbreviations: *PDK* (pyruvate dehydrogenase kinase), *PDH* (pyruvate dehydrogenase), *LDH* (lactate dehydrogenase).

3.10 The Effect of Various Melatonin Exposures on Expression of Genes Involved in Osteogenesis

To determine the effect of melatonin treatment on expression of osteogenic genes, real time RT-PCR studies were carried out. This was done so as to provide possible mechanisms underlying melatonin's induction of osteoblasts from hAMSCs and to determine the specific genes that were affected by melatonin. Treatment with 50 nM melatonin per Study Design A significantly increased the expression of the early osteogenic gene, *RUNX-2*. The exposure of melatonin for the last 2 d in the 21 d exposure increased the expression of *RUNX-2* gene 38.14 times compared to control and persisted in exposures that lasted for 5, 10, 14 and even 21 d. These increases in expression of *RUNX-2* were blocked with the addition of 1μM 4P-PDOT to the medium, suggesting the involvement of MT_2 melatonin receptors (Fig. 16A).

With respect to intermediate and late osteogenic gene expression, the expression of gene *OC* in melatonin-treated cells was not significantly increased over vehicle-treated cells. However, the levels of *OC m*RNA expression were significantly decreased in the presence of 4P-PDOT. The expression of osteocalcin was increased from 2.60-4.68 folds over control (1.00) and this increase was reversed by the addition of 4P-PDOT (Fig. 16B). The expression of *BMP-2* in hAMSCs treated with melatonin for 2, 5, 10 and 14 d, but not 21 d, showed a 2.1-3.8 fold increase over vehicle-treated cells. This increase in the expression of the mRNA was inhibited by the addition of 4P-PDOT (1μM) to the medium (Fig. 16C). Finally, the expression of *BMP*-6 and *ALP* mRNA was not statistically different in cells exposed to melatonin compared to vehicle-treated cells and the addition of 4P-PDOT was without effect on either gene (figs.16D and E).

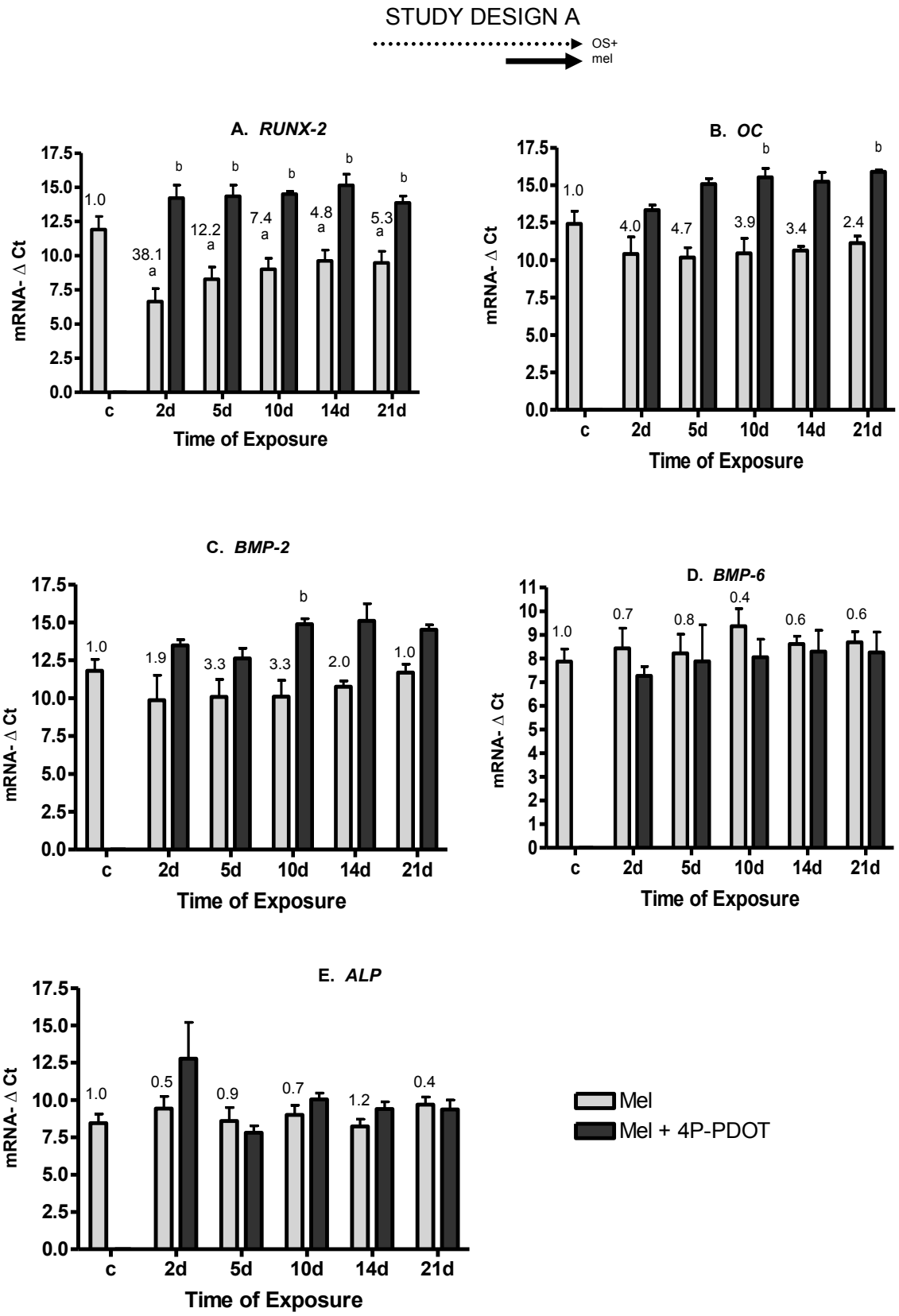

STUDY DESIGN A
OS+
mel
A. RUNX-2
B. OC
C. BMP-2
D. BMP-6
E. ALP
mRNA- Δ Ct
Time of Exposure
c
2d
5d
10d
14d
21d
Mel
Mel + 4P-PDOT

Figure 16: The Effect of Melatonin Exposure on the Expression of Genes Involved in Osteogenesis

To assess whether or not changes in genes involved in osteogenesis within differentiating hAMSCs were regulated by melatonin, real time RT-PCR analysis was carried out. The cells were cultured in $10cm^2$ dishes in OS+ medium alone (control) or in presence of 50 nM melatonin (mel) for 21 d. As described in Materials and Methods, cells were lifted in TRIZOL, total RNA was isolated and reverse transcribed into cDNA. Real-time RT-PCR analysis was carried out using primers specific for *RUNX-2*, *OC, BMP-2*, *BMP-6*, *and ALP*. The primer sequences are given in Table 1. As shown, the expression of early osteogenic gene- *RUNX-2* was significantly increased in cells treated with melatonin compared to vehicle-treated cells and this increase was blocked by 1 μM 4P-PDOT (A). The expression of gene *OC* in melatonin-treated cells was not significantly increased over vehicle-treated cells. However, the levels of *OC m*RNA expression were significantly decreased in the presence of 4P-PDOT (B). Melatonin exposure during the last 2, 5, 10 and 14 d of the 21 d OS+ exposure increased mRNA expression of BMP-2 compared to vehicle-treated cells. No increase in BMP-2 expression occurred if the melatonin-exposure was constant and lasted for full 21 d. The addition of 4P-PDOT blocked melatonin's effects (C). No melatonin-mediated or 4P-PDOT- mediated changes occurred in mRNA expression of BMP-6 (D) or ALP (E), compared to vehicle-treated cells. ΔC_t values were determined by normalization to *GAPDH* to control for variation in cDNA levels between samples. Lower ΔC_t values indicate higher gene abundance. The relative fold changes over control are denoted as numbers in the bars. Each bar represents mean ± SEM of 3-10 experiments. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test. ^{a} $p < 0.05$ compared to control, ^{b} $p < 0.05$ compared to melatonin-treated group for the same treatment time. Abbreviations: *RUNX-2* (runt related transcription factor-2), *OC* (osteocalcin), *BMP-2* (bone morphogenetic protein-2), *BMP-6* (bone morphogenetic protein-6), *ALP* (alkaline phosphatase).

4. DISCUSSION

Various studies have reported that melatonin can have a protective effect on bone by stimulating osteogenesis and preventing its degradation (Cardinali et al., 2003; Fjelldal et al., 2004; Turgut et al., 2005; Witt-Enderby et al., 2006) through a variety of mechanisms. Some of the mechanisms include- 1) inhibiting bone resorption through its effect on osteoclasts, RANKL (NF-κB ligand) and OPG (osteoprotegerin), 2) scavenging free radicals generated by osteoclasts and bone metabolism processes, and 3) stimulating the proliferation and differentiation of osteoblasts (Roth et al., 1999; Koyama et al., 2002; Suzuki and Hattori, 2002; Radio et al., 2006; Witt-Enderby et al., 2006; Suzuki et al., 2008b).

Melatonin has previously been reported to stimulate the differentiation of hAMSCs into osteoblasts when the cells were exposed to 50 nM melatonin for 10 d in presence of osteogenic medium (OS+ medium, described previously) (Radio et al., 2006). The aim of this study was to determine the critical time periods of melatonin exposure that are required for stimulating the differentiation of hAMSCs into osteoblasts and to determine the various osteogenic genes involved in the process. Mineralization, an essential component of bone differentiation process, takes place between 14 and 21 d in culture (Matsumoto et al., 1991; Eijken et al., 2005). The hAMSCs were, therefore, cultured in the OS+ medium for 21 d to induce their differentiation into osteoblasts whereby melatonin was added to the medium at different time points and for different time periods during the course of the culture. The treatment paradigm was divided into two study designs based on when melatonin was added to the OS+ medium over the period of the 21 d culture. As depicted in Fig. 5, Study Design A consisted of cells that

were exposed to 50 nM melatonin during the latter part of the 21 d culture. Study Design B consisted of cells that were exposed to 50 nM melatonin at the beginning of the 21 d culture. These two study designs were performed as such, so as to determine when melatonin exerts its maximal effect in stimulating osteogenesis- at a later stage of osteoblastic differentiation (Study Design A) or the initial phase of differentiation (Study Design B). These study designs and treatment groups would also indicate if short term or longer melatonin exposure was favorable to osteogenesis.

ALP activity is a used as a marker to assess osteoblast differentiation and its activity is reported to increase as the differentiation progresses (van Straalen et al., 1991; Bruder et al., 1997; Christenson, 1997; Jaiswal et al., 1997; Pittenger et al., 1999). In the present study it was observed that a continuous melatonin exposure for 21 d in hAMSCs increased their differentiation into osteoblasts as was measured by an increase in ALP activity, compared to the control group of cells that was devoid of melatonin exposure (Fig. 7). Continuous exposure to parathyroid hormone, PTH, is known to cause an increase bone resorption resulting in bone loss through osteoclast differentiation and increases in the RANKL: OPG ratio. An intermittent exposure, though, causes stimulation of bone formation (Hock and Gera, 1992; Locklin et al., 2003). These data suggest that the duration of exposure, continuous or intermittent, can modulate hormone effects on bone metabolism. Results from the various treatment groups in our study suggest that unlike the parathyroid hormone, melatonin needs to be administered continuously to have a positive effect on bone formation. This may hold significance in-vivo as melatonin may provide a therapy for osteoporosis that is more convenient than PTH.

Often, early biochemical events associated with the differentiation process trigger a signaling cascade culminating with transcription regulatory changes leading to differentiation of cells. For example, in keratinocytes, early exposure to calcium for 3-6 d commits most cells in culture to differentiation (Dotto, 1999). Notch, a protein involved in the regulation of osteoblast and osteoclast homeostasis (Tezuka et al., 2002; Sciaudone et al., 2003; Zamurovic et al., 2004; Engin et al., 2008) is also involved in differentiation of embryonic stem cells to neural cells where it directs a domino effect. Once it is switched on in a small group of cells, it sets off a wave of Notch activation in neighboring cells, directing them all to become cells of the nervous system (Lowell et al., 2006). To investigate whether melatonin could have a similar effect in triggering the process of differentiation, ALP activity was measured after melatonin was administered to the cells in the early stages of differentiation process and then removed from the medium (Study Design B- Fig. 7B). These results demonstrate that melatonin did not have an effect on ALP activity when added to hAMSCs and then removed, suggesting that melatonin does not have a domino effect on the differentiation processes.

Interestingly, a significant increase in ALP activity was observed when melatonin was added to the medium for 2 d towards the end of the 21 d in OS+ culture, compared to the cells treated only with OS+ medium for 21 d (Study Design A- Fig. 7A). However, no significant affect on the ALP activity in cells treated with melatonin for 5, 10 or 14 d towards the end of the 21 d culture in OS+ medium compared to control cells. These results were similar to the study reported by Roth et al (1999) which suggested that MC3T3 cells must first undergo differentiation before they become responsive to melatonin. Although our 21 d continuous melatonin treatment does not support this

hypothesis, it is possible that the hAMSCs were in an advanced stage of differentiation due to the osteogenic medium (OS+) effects on the intracellular machinery required for melatonin to have its effect (melatonin was added on the 19th d of culture in osteogenic medium). These results underscore the importance of both timing and duration of melatonin on inducing osteoblast differentiation from hAMSCs.

The osteoblast lineage begins with multi-potent mesenchymal stem cells, located in the bone marrow. Once they are committed to a lineage (i.e.,. osteoblasts, adipocytes or chondrocytes), the stem cells begin to divide to give rise to another stem cell and an immature osteo-progenitor cell. This process is called self renewal. The immature osteo-proginetor cell matures and undergoes intensive proliferation and further differentiation to form pre-osteoblasts. Differentiation of pre-osteoblasts gives birth to mature osteoblasts that have limited replicative potential. The terminal stage of this differentiation process is an osteocyte which lays new bone (Thomas and Kansara, 2006).

From our study it can be hypothesized that there is an increase in differentiation of hAMSCs into osteoblasts (as measured by an increase in ALP activity) when the cells were exposed to melatonin either before the differentiation is initiated (21 d), or at a later stage of differentiation, when the machinery required for melatonin-induced differentiation was induced by a 19 d pre-exposure to osteogenic medium. Possibly, melatonin does not have an effect on the intermediate stages of hAMSC differentiation as measured by an increase in ALP activity. The intermediate stages of differentiation of stem cells are marked by a sharp increase in proliferation In our system we see no change in the proliferation of stem cells following melatonin exposure (Fig. 13), suggesting that melatonin acts to modulate different stages of differentiation rather than

proliferation. These results are consistent with cellular differentiation as a whole where high stages of differentiation are most often associated with low levels of proliferation. The increase in ALP activity was not observed, when melatonin was added for less than 2 d towards the end of the 21 d culture. These findings suggest that the time of melatonin exposure was not long enough on the MT_2 melatonin receptor to impact on downstream signaling processes (Fig. 7B). As mentioned before, exposure and then removal of melatonin from the medium (Study Design B), resulted in no increase in ALP activity, suggesting that continuous melatonin treatment is necessary for the increase in differentiation process.

This biphasic response to melatonin could also be a result of the cyclic expression of ALP throughout the 21 d treatment. In primary mouse calvarial cells and embryonic stem cells, ALP expression increases first and then decreases in cells treated with osteogenic medium (zur Nieden et al., 2003; Siddappa et al., 2009). In a recent study using rat mesenchymal stem cells cultured in osteogenic medium, it was shown that both ALP activity and its mRNA levels were decreased in the early (0-8 d) and late stages (> 16 d) of differentiation, compared to the intermediate stage (8-16 d) (Nakamura et al., 2009). Although not tested in this study, it can be speculated that during the intermediate stage, levels of ALP expression induced by the osteogenic medium alone were maximal such that melatonin was not able to increase them further. More experiments using osteogenic medium alone are needed to investigate the reason for this response and to determine if there are cyclic changes in the ALP expression levels as the differentiation progresses.

Studies were carried also out to determine how the level of pre-differentiation of cells induced by the osteogenic medium alone affected the 2 d melatonin increase in ALP activity (Fig. 8). These experiments were performed by varying the time period of osteogenic medium exposure before adding melatonin for 2 d. The data show that there is a critical time of osteogenic pre-exposure required (i.e.,. 19 d) before a 2 d melatonin can induce its highest effect on ALP activity. These findings corroborate our previous hypothesis that melatonin can increase the ALP activity when cells were exposed to it in later stages but not in intermediate stages of differentiation.

To further support the hypothesis that melatonin, added in combination with osteogenic medium enhances hAMSC differentiation into osteoblasts, calcium deposition was measured in addition to ALP activity. These data show that melatonin-induced increases in ALP activity were associated with increases in calcium deposition (figs. 11A and B). Calcium deposition as judged by alizarin red staining was distributed evenly through the culture and not localized to a few cells (Fig. 12). An increase in calcium deposition or mineralization is one of the most important markers of bone formation (Matsumoto et al., 1991; Jaiswal et al., 2000; Bancroft et al., 2002; Titorencu et al., 2007). Thus, in hAMSCs, a 21 d continuous melatonin exposure enhances cellular differentiation. Although the results show that 2 d melatonin exposure towards the end of the 21 d OS+ exposure period also causes an increase in ALP activity, this did not translate to enhancement in calcium deposition (Fig. 11A). Calcium deposition is one of the final markers of bone formation, and as explained further in the following paragraphs, it may be a better marker to study bone formation than ALP activity. The increase of melatonin-induced ALP activity and calcium deposition were inhibited in the presence of

the MT_2 melatonin receptor antagonist 4P-PDOT, suggesting that MT_2 melatonin receptors were involved in this process (Fig. 7D and 11C).

Melatonin receptors mainly couple to G_i proteins and, upon stimulation they inhibit intracellular cyclic AMP accumulation (Witt-Enderby and Dubocovich, 1996; von Gall et al., 2002; Sethi et al., 2008). As shown in Fig. 10, hAMSCs first treated with melatonin for 2 d or 21 d (Study Design A) and then re-challenged with melatonin (20 min) did not result in an inhibition of forskolin induced cAMP accumulation, indicating desensitized melatonin receptors. To determine whether or not melatonin binding sites were modified following hAMSCs differentiation into an osteoblast, 2-[^{125}I]-iodomelatonin saturation analysis was performed. Results indicate that differentiating hAMSCs express low levels melatonin receptor binding sites ($\leq$ 1.5 fmol/mg protein), however they were not significantly modulated by the different melatonin exposures, except for the 10 d melatonin exposure. Exposure to melatonin for 10 d towards the end of the 21 d treatment demonstrated an increase in 2-[^{125}I]-iodomelatonin binding. This increase in binding could reflect changes in the MT_2 – G protein coupling, since 2-[^{125}I]-iodomelatonin is an agonist radioligand and its binding to melatonin receptors could be modulated by the state of MT_2 receptor/ G-protein coupling. These data indicate that the functional sensitivity of MT_2 melatonin receptors expressed in differentiating hAMSCs may underlie the melatonin-induced hAMSCs differentiation into osteoblasts and corroborates previous results shown by Radio et al (2006), where it was reported that melatonin-mediated decreases in cAMP were attenuated at the peak of hAMSCs differentiation into osteoblasts. It was also shown in this study that the loss of coupling to cAMP-dependent pathways was accompanied by a gain in MAPK function through β-

arrestin scaffolding to MT_2 receptor in these same cells (Radio et al., 2006). Similar mechanisms have been reported for PTH (parathyroid) receptors expressed in HEK cells, where ERK 1/2 activation occurred through both a cAMP dependent pathway as well as a β-arrestin dependent pathway that depended upon the duration of PTH exposure (Gesty-Palmer et al., 2006).

There have been many studies reported on melatonin's action on mitochondria and its functions. Mitochondria generate most of the cell's supply of adenosine triphosphate (ATP) through the electron transport chain. The electron transport chain constantly leaks a small amount of electrons that cause oxidative damage to DNA, proteins, and lipids through the production of ROS (reactive oxygen species) (Adam-Vizi and Chinopoulos, 2006). Melatonin is a powerful antioxidant and free radical scavenger that directly scavenges ROS (Reiter et al., 2001). Melatonin's effect on the mitochondria include increased membrane fluidity, more efficient activity of the electron transport chain, reduced oxidative stress and increased mitochondrial membrane potential (Acuna-Castroviejo et al., 2007). Studies have shown that mitochondrial function is related to the differentiation process (Duguez et al., 2002; Cho et al., 2006; Chen et al., 2008a). As stem cells lose pluripotency and commit to a lineage, the expression of mtDNA (mitochondrial DNA) transcription and replication factors is upregulated and the number of mitochondria and mtDNA copies/ cell increases (Facucho-Oliveira and St John, 2009). A study using human mesenchymal stem cells has reported that an increase in differentiation of the cells into osteoblasts caused an increase in the mRNA levels of genes involved in mitochondrial biogenesis- *mtTFA, Pol-γ*, and *PGC-1α* (Chen et al., 2008a) . There was also a concurrent decrease in lactate production rate, glycolytic

enzyme PDK and increase in PDH, the enzyme that is responsible for converting pyruvate into acetyl CoA to enter the tricarboxylic acid cycle and aerobic metabolism. It was proposed that on induction of differentiation, there is a "metabolic shift" which reprograms the bioenergetic properties of hAMSCs from anaerobic glycolysis to mitochondrial respiration to meet the higher energy demand of differentiated osteoblasts (Chen et al., 2008a). In our study, the levels of the genes involved in mitochondrial biogenesis and glycolysis did not change with a 21 d melatonin treatment (Fig. 14 and 15). While this does not indicate whether melatonin treatment alters the mitochondrial function or if it induces the production of antioxidant enzymes in these cells, this was the first study looking at the effect of melatonin on mitochondrial biogenesis in differentiating hAMSCs. Perhaps, the levels of the genes involved in mitochondrial biogenesis plateaued during hAMSC differentiation with osteogenic medium (OS+) alone as shown in another study (Chen et al., 2008a), such that no further effect of melatonin could be observed. Also, it is possible that melatonin does participate in the "metabolic shift" of these cells from anaerobic glycolysis to mitochondrial respiration because there was no change in the mRNA expression levels of genes involved in cellular metabolism like *PDH* and *PDK*.

The differentiation of osteoblasts from hAMSCs is controlled by a variety of osteogenic genes. The most important of them is *RUNX-2*, which is a master regulator of many other osteogenic genes (Ducy et al., 1997). *RUNX-2* can directly stimulate transcription of *OC* by binding to specific enhancer regions of the gene (Kim et al., 2007). Osteocalcin and other proteins allow osteoblasts to mineralize collagen I-rich matrix to form bone (Kwok et al., 2009). A mutation of this gene in mice leads to a

skeletal system that is completely devoid of ossification (Komori et al., 1997). Bone morphogenetic proteins (BMPs) are a part of the TGF-β superfamily of ligands. As shown in Fig. 3, BMP-2 binds to a TGF- β type II transmembrane receptor. The type II receptor, a serine/ threonine receptor kinase, phosphorylates the TGF- β type I receptor and forms a complex. These complex results in phosphorylation of downstream regulators called R-smads which complex with Co-smads. This complex translocates into the nucleus to regulate the transcription of target osteogenic genes (e.g. *RUNX-2*) (Lian et al., 2006; Huang et al., 2007). As shown in Fig. 3, another transcription factor called The gene, osterix is activated downstream of *RUNX-2*, which is indispensable for bone development (Matsubara et al., 2008). In osterix null mice, an absence of bone formation occurs at the embryonic stage (Nakashima et al., 2002). *BMP-2* treatment has been reported to cause an increase in expression of *RUNX-2* mRNA (Lee et al., 2000). BMP-2 can also signal in a Smad-independent manner and can activate the MAPK pathway which can in turn regulate *RUNX-2* expression in osteogenic cells (Lai and Cheng, 2002; Xiao et al., 2002; Huang et al., 2007). BMP-2 also controls osteogenesis through Wnt/ beta-catenin signaling (Fig. 3) (Rawadi et al., 2003).

Real time RT-PCR studies on hAMSCs treated with melatonin or melatonin and 4P-PDOT demonstrated that *RUNX-2* mRNA expression was increased by melatonin (Fig. 16A). Although not statistically significant, we also observed a consistent increase in the mRNA expression levels of *BMP-2* and *OC* with the treatment of melatonin (figs. 16B and C) that was blocked by the use of 4-P-PDOT at all time periods studied, indicating that MT_2 receptors were involved in the process. Unexpectedly, the mRNA

expression of *ALP* did not change on melatonin treatment. The possible reasons are outlined in the following paragraph.

Based on the results, we suggest that melatonin, acting through MT_2 melatonin receptors, increases the differentiation of hAMSCs as measured by an increase in ALP activity and through an increase in mRNA expression of the osteogenic genes *RUNX-2, BMP-2* and *OC*. From the results of this study, it is hypothesized that there are at least 2 different signal transduction pathways involved in melatonin-mediated differentiation of hAMSCs into osteoblasts, as depicted in Fig. 17. The pathway (marked B in schematic) shows that MAPK activity is increased through MT_2 receptor/ β-arrestin scaffold formation and this acts to increase ALP activity. MT_2R/ β-arrestin scaffolds only form following MT_2 receptor desensitization by chronic melatonin exposure (Radio et al., 2006). Though not modulated via transcriptional events, the increase in ALP activity could be due to translational or posttranslational processing to increase ALP activity.

The second pathway (denoted A in schematic) hypothesized by this study also involves the MAPK pathway but involves sensitive MT_2 receptors, that is, melatonin receptors capable of inhibiting forskolin induced cAMP formation when activated by melatonin. It is suggested that the activation of G_i protein due to binding of melatonin to MT_2 receptors inhibits adenylyl cyclase and PKA and dephosphorylation of raf-1, which causes activation of MEK(1/2) and ERK(1/2). Melatonin has been previously reported to increase phosphorylation of these proteins through an inhibition of PKA in MT_1-CHO cells (Witt-Enderby et al., 2000). PKA acts to inhibit Raf-1 or Raf-B that are activators of MEK (1/2) (Burgering and Bos, 1995). Activation in ERK (1/2) can directly phosphorylate sites on the *RUNX-2* transcription factor causing an increase in

transcription of other osteogenic genes (Xiao et al., 2000; Franceschi et al., 2007). *OC* and *BMP-2* are genes whose expression is controlled by *RUNX-2*. After translation, the BMP-2 protein can act as an autocrine/ paracrine factor and stimulate transmembrane TGF-β receptors and activate the MAPK pathway (Derynck and Zhang, 2003). BMP-2 has been reported to activate various matrix metalloproteinases (MMP) in some studies (Nakashima and Tamura, 2006; Tang, 2008), and the inhibition of MMP caused a decrease in melatonin-induced increase in ALP activity in hAMSCs (Radio et al., 2006).

There are reasons why two separate pathways are hypothesized for the effect of melatonin on differentiation of hAMSCs. The increase of ALP activity following 2 d or 21 d melatonin treatment (Study Design A) was not correlated with the increase in mRNA expression of osteogenic genes that were increased at all time periods of melatonin exposure. This, in itself, may not be substantial evidence for separate pathways because gene expression precedes protein expression in a cell, however, the complete lack of the trend (observed with ALP activity) points towards a different pathway. Secondly, the mRNA levels of the osteogenic genes *RUNX-2, BMP-2* and *OC* were increased at time periods of melatonin treatment when the melatonin receptors were sensitive as well as desensitized (with respect to a decrease in forskolin induced cAMP activity), whereas the ALP activity was increased only with those time periods of melatonin exposure that caused the melatonin receptors to desensitize with respect to cAMP signaling. Lastly, the expression of *ALP* mRNA did not change with melatonin treatment, but its upstream regulators (*RUNX-2 and BMP-2*) and downstream markers (ALP activity) were increased compared to the control cells following 2 d and 21 d time exposures. From this, it can be hypothesized that there is a missing connection between

the upstream and downstream markers and this is possibly due to their signaling *via* different pathways. As previously shown (Radio et al., 2006), the increase in ALP activity is dependent upon MEK and ERK1/2 activation as inhibition of MEK1/2 by a MEK inhibitor blocked completely the melatonin-induced increases in ALP activity in hAMSCs (Radio et al., 2006).

It is hypothesized that co-modulation of MAPK through different pathways ensures appropriate intracellular signals to enhance hAMSC differentiation. Because the endogenous regulation of melatonin biosysthesis, both systemic and in the bone marrow, is chronobiotic in nature with high levels of melatonin occurring during the period of darkness which persist for at least 8 h each night, then differential modulation of melatonin receptor sensitivity states within the bone may serve to direct appropriate intracellular pathways to maintain healthy bone. In this study, an increase calcium deposition, which is a final result of osteogenesis did not correlate with an increase in ALP activity. The increase in ALP activity also did not correlate with the expression of various osteogenic genes. From this, it is suggested that ALP activity may not be the best indicator for differentiation. Mineralization may be a better marker as it can provide an indication of final stages of differentiation and is less likely to be influenced by various factors to which a protein like ALP could be susceptible.

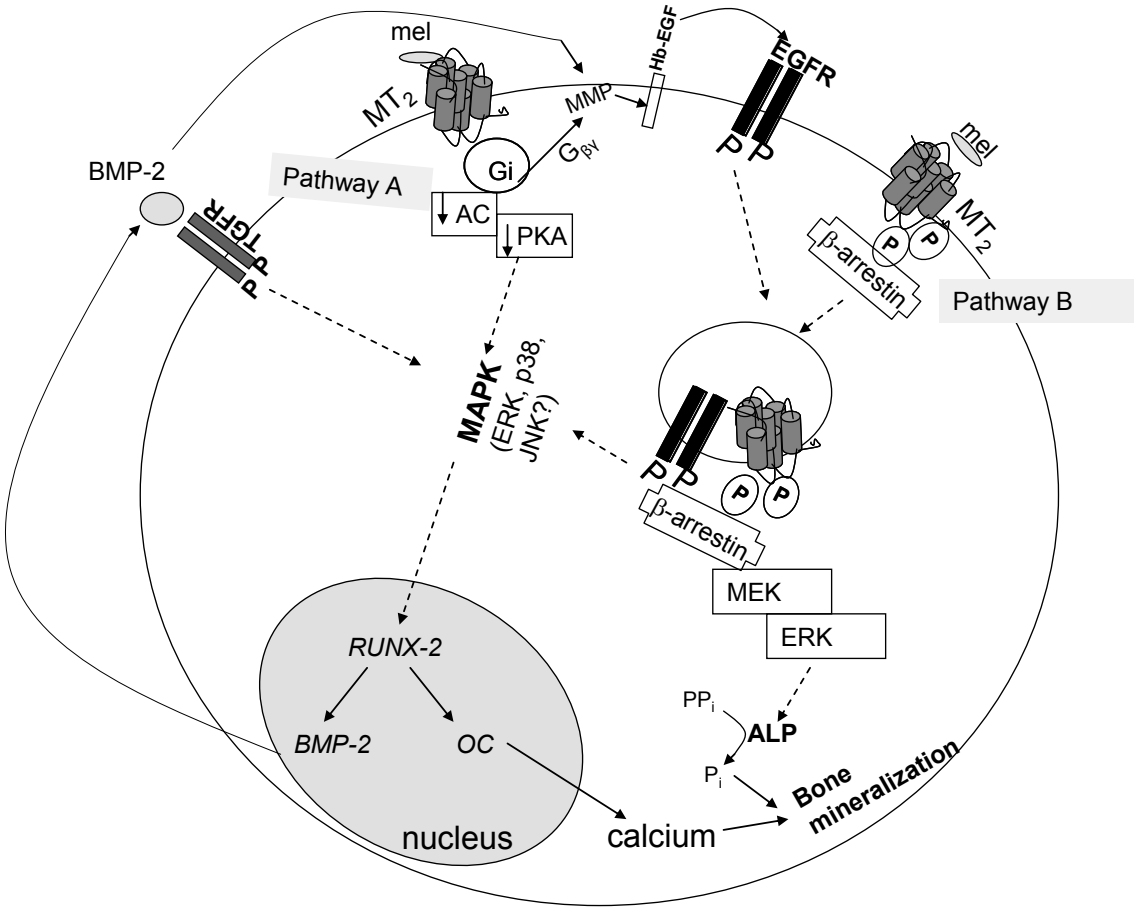


Figure 17: Proposed Mechanism of Melatonin's Action within a Differentiating Human Adult Mesenchymal Stem Cell into an Osteoblast

In the present study it is proposed that melatonin could affect the differentiation of hAMSCs into osteoblasts through two pathways denoted as A and B in the schematic. As shown in pathway A, melatonin, through an activation of MT_2Rs leads to an inhibition of adenylyl cyclase, decreases in cAMP and decreases in PKA which then promotes the activation of MAPK (ERK1/2, p-38 or JNK). As shown in pathway B, upon chronic melatonin exposure, MT_2Rs desensitize and form scaffolds with β-arrestins/Gi/MEK/ERK1/2) leading to ALP activation in the cytoplasm. Both pathways converge on MAPK (ERK1/2, p-38 or JNK), which then increases the expression of the transcription factor *RUNX-2* in the nucleus. The activation of *RUNX-2* increases the mRNA expression of osteogenic genes *OC* and *BMP-2*. The BMP-2 protein upon translation can act as an autocrine/ paracrine factor to also activate MAPK via Smad-independent signaling via pathway A. BMP-2 may also contribute to the activation of pathway B by activating MMPs resulting in HB-EGF shedding and activation of EGFR. **[**Abbreviations: EGFR (epidermal growth factor receptor), HB (heparin bound), EGF

(epidermal growth factor), MEK1/2 (MAP-ERK kinase), ERK1/2 (extracellular signal-regulated kinase), MMP (matrix metalloproteinase), ALP (alkaline phosphatase), *RUNX-2* (Runt-related transcription factor 2), *BMP-2* (bone morphogenetic protein-2), *JNK* (c-Jun N-terminal kinases).

5. CONCLUSION

The findings from this study demonstrate that melatonin enhances bone formation through the enhancement of osteoblast differentiation in a clinically relevant human adult mesenchymal stem cell line. Various treatment times of melatonin exposure caused variable changes in different markers of osteogenesis and a continuous (21 d) treatment resulted in an increase in calcium deposition. These findings suggest that a continuous melatonin treatment might be most effective at preventing or treating osteoporosis. These results also reinforce the prominent role that MT_2 melatonin receptors play in bone stem cell differentiation and through the mitogen activated protein kinase cascade. As no major side effects have been reported with melatonin administration, drugs targeted at MT_2 melatonin receptors to modulate its sensitivity states may provide novel, safe, alternative therapies to treat osteoporosis. Furthermore, seeing that the risk of osteoporosis increases in women with age and for nightshift workers (two conditions that result in low circulating levels of melatonin), perhaps directed therapies to replenish night time melatonin levels back to normal could prevent against bone loss.

Chapter 2
A Study on C-Terminal Domains of MT_1 AND MT_2 Melatonin Receptors

6. INTRODUCTION

Melatonin is released from the pineal gland during the hours of darkness. Melatonin's levels in the body are decreased in a variety of diseases, e.g. breast, prostate, lung and colorectal cancer, osteoporosis, insomnia, seasonal affective disorder and Alziemer's disease (Ekmekcioglu, 2006). Melatonin often exerts its effects via G-protein coupled melatonin receptors, MT_1 and MT_2 (Reppert et al., 1994; Reppert et al., 1995), however, it can modulate quinone reductase-2 enzyme activity via a melatonin binding site, *MT_3* (Tan DX et al., 2007). An orphan receptor (GPR50) was also identified, which has a 45% homology to the melatonin receptors (Reppert et al., 1996; Drew et al., 1998).

MT_1 and MT_2 receptors mainly couple to pertussis toxin-sensitive G_i proteins (Carlson et al., 1989; Brydon et al., 1999), and inhibit intracellular cAMP accumulation followed by a decline in PKA activity and CREB (cAMP regulatory element-binding protein) phosphorylation (Witt-Enderby and Dubocovich, 1996; von Gall et al., 2002; Dubocovich et al., 2003). Similar to other GPCRs, the MT_1 and MT_2 have seven transmembrane (TM) domains, connected by three extracellular and three intracellular loops, and consist of 350 and 362 amino acids respectively (Mazna et al., 2004; Hardeland, 2009). Homology models of the melatonin receptors are based on the crystal structure of rhodopsin (Palczewski et al., 2000). The extracellular loops may be involved in ligand recognition, and the intracellular loops may participate in G-protein binding (Drew et al., 1998; Mazna et al., 2005). Based on the knowledge of the rhodopsin structure, this study sought to understand the function and regulation of melatonin

receptors through construction of two mutants in the C-terminal tails of the MT_1 and MT_2 receptors (Fig.18).

In the first mutation, a cysteine residue in the C-terminal tail was mutated to alanine (MT_1C7.72A and MT_2C7.77A). This site is essential for the G-protein binding of the rhodopsin receptor. The cytoplasmic surface of rhodopsin consists of four intracellular loops. The first, second and third intracellular loops connect adjacent transmembrane helices. The fourth intracellular loop is formed by attachment of palmitoyl groups to two cysteine residues (Cys 322, and Cys 323) in the C-terminal region, and inserting it into the lipid bilayer (Ovchinnikov Yu et al., 1988; Moench et al., 1994). It has been suggested that the fourth intracellular loop interacts directly with G_t, and plays a role in the regulation of $G_{t\beta\gamma}$ subunit (Ernst et al., 2000). Palmitoylation has been reported for other GPCRs like human luteinizing hormone receptor, where the palmitoylation state of the receptor governs the accessibility of the receptor to beta-arrestins to regulate receptor internalization (Munshi et al., 2001). Mono-, bis- or tris-palmitoylation of the cysteine residues of the C-tail also occurs in β-adrenergic receptors, α-adrenergic receptors, muscarine acetylcholine receptors, vasopressin receptors, dopamine receptors, bradykinin receptors and many others (O'Dowd et al., 1989a; Kennedy and Limbird, 1994; Qanbar and Bouvier, 2003). Palmitoylation, or addition of 16-carbon saturated fatty acid, palmitic acid to the receptors is a part of post-translational modifications of GPCRs (Chini and Parenti, 2009). Although the palmitoylation can exist in intracellular loops of the GPCRs, it is most common at the C-terminal (Qanbar and Bouvier, 2003).

Addition of the palmitate could have many functions in a receptor. It can be involved in plasma membrane localization of the receptor. Blocking the palmitoylation reduced the number of receptors reaching the cell surface in TSH receptor, δ-opioid receptor and histamine H2 receptor (Tanaka et al., 1998; Fukushima et al., 2001; Petaja-Repo et al., 2006). Palmitoylation can be involved in receptor-G-protein coupling (O'Dowd et al., 1989b). In the muscarinic acetylcholine receptor M2, the lack of palmitoylation reduced the ability of G proteins to activate (Hayashi and Haga, 1997). In human somatostatin receptor type 5, the mutation of the palmitoylation sites led to a reduced coupling to adenylate cyclase (Hukovic et al., 1998). The effects of palmitoylation on signaling are not universal. Human A_1 adenosine receptor and dopamine D_1 receptor are some of the receptors where the signaling is not affected by lack of palmitoylation (Jin et al., 1997; Gao et al., 1999). The C-terminal tails of the MT_1 and MT_2 receptors contain a single cysteine residue, which could be a possible palmitoylation site and may form a fourth intracellular loop. Mutation of this cysteine within the melatonin receptors might prevent the fourth intracellular loop from forming, which may affect receptor function.

In the second mutation, the C-terminal tail in the MT_1 and MT_2 receptors was truncated (MT_1Y7.64 and MT_2Y7.64). The role of the C-terminal tail in receptor phosphorylation and subsequent beta-arrestin binding is well-known. Beta-arrestins are proteins that are involved in the desensitization of most GPCRs (DeWire et al., 2007) , including MT_1 (Bondi et al., 2007) and MT_2 receptors (Gerdin et al., 2003a). In most GPCRs, upon prolonged agonist stimulation, GPCRs undergo conformational changes that are recognized by the family of GPCR kinases (GRKs) that phosphorylate the serine,

threonine, and tyrosine residues on their intracellular loops and C-terminal tails (Premont and Gainetdinov, 2007; Kovacs et al., 2009). Beta-arrestin recruitment to these phosphorylated amino acids stearically blocks the binding of the receptor to G proteins, thus causing acessation of G protein signaling (desensitization), despite the continued presence of the receptor-activating agonist. The arrestins form a complex which is transferred to clathrin coated pits for endocytosis, recycling and degradation (DeWire et al., 2007). Based on this information, it was hypothesized that truncation of the C-terminal tail of the melatonin receptors would remove a majority of these phosphorylation sites and impair receptor internalization.

The mutations were named according to the scheme described by Ballesteros and Weinstein. The most conserved residue within each transmembrane domain was designated number 50 (in this case alanine) and is preceded by the number of the TM domain (Ballesteros J and Weinstein., 1995).

Because it has been shown that desensitization/ internalization processes drive cellular events induced by melatonin (Radio et al., 2006; Bondi. et al., 2007) and beta-arrestin scaffolds modulate melatonin receptor function (Gerdin et al., 2003a; Bondi. et al., 2007), the focus of this study was to determine if the above mentioned domains were involved in melatonin receptor desensitization and internalization processes.

7. MATERIALS AND METHODS

7.1 Cell Culture and Transfections

COS-7 cells were grown as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 2 mM glutamine. The cells were grown to 60–70% confluence and transiently transfected with MT_1 or MT_2 wild-type (WT) or mutant constructs using genePORTER transfection reagent (Genlantis, San Diego, CA), as per manufacturer's instructions.

7.2 Construction of Mutant Plasmids

Two mutations were made to the genes encoding human MT_1 or MT_2 receptors (Fig. 18) using site-directed mutagenesis (Quikchange ll; Stratagene, La Jolla, CA), according to manufacturer's instructions. In the first mutation, a cysteine residue in the C-terminal tail of each receptor was converted to alanine using primer pairs (MT_1C7.72A: forward = GAATTATAGTCTCGC TCGCTACAGCCAGGGTGTTC; reverse = GAACACCCTGGCTGTAGCGAGCGAGACTATAATTC) and (MT_2C7.77A: forward = GAACCCACGGCACGCCATTCAAGATGCTTC; reverse = GAAGCATCTTGAATGGCGTGCCGTGGGTTC). This substitution removes the putative palmitoylation site of the C-terminal tail, an important component for signaling in some other GPCRs. In the second mutation, the C-terminal tail of each receptor was truncated, deleting all potential phosphorylation sites in the C-terminal tail using primer pairs (MT_1Y7.64: forward = CCAAAATTTCAGGAAGGAATAAAGGAGAATTATAGTCTCGCTC; reverse = GAGCGAGACTATAATTCTCCTTTATTCCTTCCTGAAATTTTGG) and (MT_2Y7.64: forward = CCAAAACTTCCGCAGGGAATAAAAGAGGATCCTCTTG;

reverse = CAAGAGGATCCTCTTTTATTCCCTGCGGAAGTTTTGG). All constructs included a green fluorescent protein (GFP) tag at the C-terminal region. Gene products for MT_1 WT, MT_2 WT, MT_1Y7.64 and MT_2Y7.64 were each cloned into pcDNA3 (Invitrogen), and the gene products for MT_1C7.72A and MT_2C7.77A were each cloned into pcDNA1 (Invitrogen, Carlsbad, California). All mutants were confirmed by sequencing.

7.3 Radioligand Binding Assays

Saturation binding assays were performed using 2-[^{125}I]-iodomelatonin on whole cell lysates. The COS-7 cells were grown and transfected with the MT_1 or MT_2 WT or mutant constructs in 10-cm^2 culture dishes, as described in the methods section. On the third day after transfection, cells were washed twice with 2 mL of phosphate-buffered saline (PBS) and collected in buffer (0.25 M sucrose, 10 mM potassium phosphate, 1 mM EDTA, pH 7.4) and pelleted by centrifugation (500 x *g*, 5 min). The cells were then resuspended in cold Tris-HCl (50 nM, pH 7.4) buffer containing 1 X complete mini protease inhibitors (Roche Diagnostics, Mannheim, Germany), and aliquoted into tubes containing concentrations of 2-[^{125}I]-iodomelatonin (2,200 Ci/mmole; NEN/DuPont, Boston, MA) ranging from 0-1 nM in the absence or presence of 1 μM melatonin. The reactions were incubated for 1 h at room temperature and then terminated by ice-cold Tris-HCl buffer (50 nM, pH 7.4). Filtration was performed using glass fiber filters (0.22 μM), presoaked in 0.5% polyethylinimine, and using a Brandel Cell Harvester. Each filter was washed twice with 5 ml Tris-HCl buffer. Radioactivity was counted using a gamma counter. Saturation analysis was performed using GraphPad Prism Software© (GraphPad Inc., San Diego, CA). The affinity (K_D) and density (B_{max}) of 2-[^{125}I]-

iodomelatonin binding were determined for each experiment performed and then averaged. Statistics were performed using one-way ANOVA followed by a Newman-Keuls post hoc t-test where significance was set at $p<0.05$.

7.4 cAMP Accumulation Assays

The cAMP accumulation assays were carried out by enzyme immuno-antibody according to manufacturer's instructions. COS-7 cells were transfected with MT_1 or MT_2 WT or mutant constructs in 12-well plates. On the third day after transfection, the cell medium was aspirated, and the cells were incubated in serum-free media containing either 30 μM rolipram alone (basal), 30 μM rolipram and 100 μM forskolin (maximal accumulation) or 30 μM rolipram, 100 μM forskolin and 10 nM melatonin. cAMP accumulation was expressed as a percentage of forskolin response within each group. Statistical analysis was performed using commercial software (graphpad prism®, GraphPad Prism, Inc.), whereby the maximal efficacy of melatonin to inhibit forskolin-induced cAMP accumulation was compared against the maximal forskolin response within each group.

7.5 Confocal Microscopy

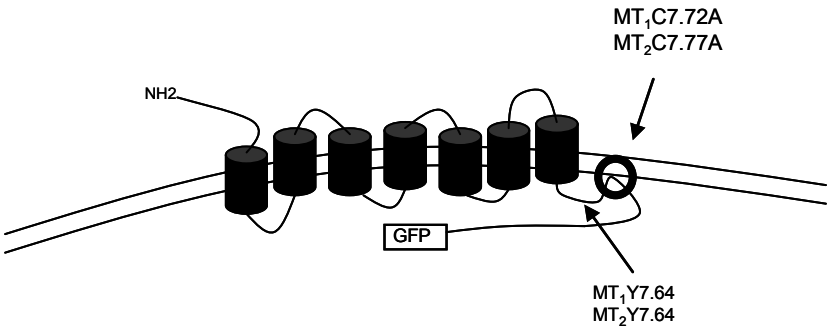
The cells were grown and transfected with MT_1 or MT_2 WT or mutant constructs, as described in the methods section. On the third day after transfection, the cells were lifted with trypsin and seeded on sterile coverslips for 24 h in 6-well plates. Cells were incubated in serum-free media for 5 h at 37°C and incubated with 1 μM melatonin or vehicle (0.001% ethanol) for 1 h. The cells were then washed twice with PBS and fixed with 2% paraformaldehyde for 15 min. Coverslips were mounted on slides and then

visualized under oil immersion at 1000× using a Leica TCS-SP2 confocal laser microscope (Leica Microsystems, Inc., Confocal Division, Exton, PA).

7.6 Radiolabeling with [^{3}H] Palmitic Acid

Radiolabeling of MT_1 WT and MT_2 WT receptors with [^{3}H] palmitic acid was carried out as previously described (Das et al., 2007). COS-7 cells were grown in six-well plates until the cells reached 60–80% confluence. Cells were then transfected with the MT_1 or MT_2 WT constructs. Twenty-four hours after transfection, cells were labeled with 50 μCi/mL 9,10-[^{3}H] palmitic acid for 16 h in the same media with reduced serum (3% FBS). Cells were then washed once with PBS, and scraped in cold RIPA buffer (Boston Bioproducts, Worcester, MA). Aliquots of cell lysate were added to scintillation vials and counted in a liquid scintillation counter or used in a cell proliferation assay. The amount of [^{3}H] palmitic acid counts were expressed as fmol/total cell count. Cell proliferation assay was performed on the scraped cells using one solution cell proliferation assay kit (Promega, Madison, WI) as per manufacturer's instructions.

A.



B

Y R R I I V **S** L C **T** A R V F F V D **S S** N D V A D R V K W K P **S** P L M **T** N N N
V V K V D **S** V K **S T T** F R V R W **T** R C A R P R **S** Q M **S** G K

MT_1WT

Y K R I L L A L W N P R H C I Q D A **S** K G **S** H A E G L Q **S** P A P P I I G V Q H
Q A D A L P

MT_2WT

Figure 18: Details of the C-terminal Mutations

(A) Diagram representing the site of the C-terminal mutations in MT_1 and MT_2 melatonin receptors. (B) Amino acid sequence of the C-terminal tails of the MT_1WT and MT_2 WT receptors. In mutants MT_1Y7.64 and MT_2Y7.64, all the above amino acids were removed in their respective receptor type to remove the putative phosphorylation sites in the C-tail (the amino acids represented in bold). In mutants MT_1C7.72A and MT2C7.77A, a cysteine residue in the respective C-tails was mutated to alanine (the underlined cysteines in the above sequence).

8. RESULTS

8.1 Effect of the C-terminal mutations on Affinity and Density of 2-[^{125}I]-Iodomelatonin Binding to MT_1 and MT_2 Melatonin Receptors Expressed in COS-7 cells

To determine if the mutations in the MT_1 and MT_2 receptors caused any changes in their affinity and density compared to WT receptors, saturation binding was carried out on COS-7 cells transfected with the various constructs. The results revealed that the affinity and density of 2-[^{125}I]-iodomelatonin for the mutant receptors was similar to the affinity and density obtained for WT type receptors (Table 2).

Receptor	K_D (pM)	B_{max} (fmol/mg protein)
MT_1WT	80 ± 20	0.5 ± 0.2
MT_1Y7.64	76 ± 16	0.5 ± 0.1
MT_1C7.72A	60 ± 23	14 ± 11
MT_2WT	73 ± 8	0.9 ± 0.4
MT_2Y7.64	56 ± 8	0.3 ± 0
MT_2C7.77A	75 ± 38	0.7 ± 0.2

Table 1: Saturation Binding Analysis of 2-[^{125}I]-Iodomelatonin Binding to Mutant and MT_1 WT and MT_2 WT Receptors

Saturation binding was performed on whole cell lysates of COS-7 cells transiently expressing MT_1 WT and MT_2 WT receptors as well as their respective mutants. The affinity (K_D) or B_{max} values for 2-[^{125}I]-iodomelatonin binding were not significantly different between WT and mutant receptors. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test. Each value represents the mean ± SEM of 3-5 independent experiments.

8.2 Effect of C-terminal Mutations on Melatonin-Mediated Inhibition of Forskolin-Induced cAMP Accumulation in Naive COS-7 cells Expressing MT_1 and MT_2 Receptors

To determine if the mutations affected melatonin-mediated decreases in forskolin-induced cAMP accumulation, cAMP assays were carried out in naive cells (i.e.,. cells never exposed to melatonin). As shown in Fig. 19, melatonin (10 nM) inhibited forskolin-induced cAMP accumulation in COS-7 cells expressing the WT melatonin receptors. However, no inhibition of forskolin-induced cAMP accumulation by melatonin (10 nM) occurred in COS-7 cells expressing either of the MT_1 or of the MT_2 receptor mutants.

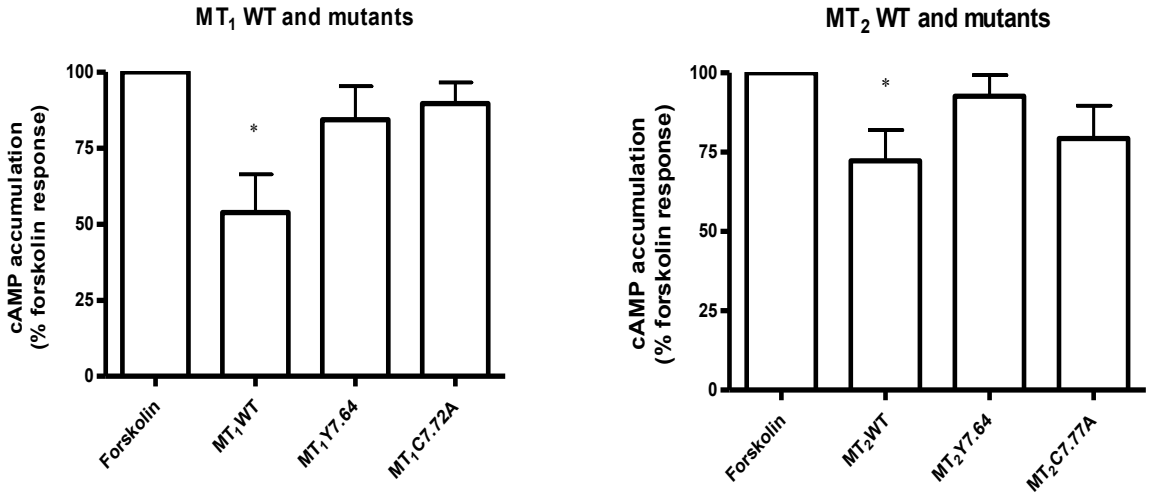


Figure 19: Melatonin Mediated Inhibition of Forskolin-induced cAMP Accumulation in COS-7 cells Transiently Expressing WT or Mutant Melatonin Receptors

As shown, a 20 min exposure to 10 nM melatonin inhibited forskolin-induced cAMP accumulation when compared to the forskolin response alone. By contrast, expression of mutant MT_1 (MT_1C7.72A, MT_1Y7.64) or MT2 (MT_2C7.77A, MT_2Y7.64) receptors within COS-7 cells followed by a 20 min exposure to melatonin did not result in an inhibition of cAMP accumulation induced by 100 μM forskolin. Each bar graph represents the mean ± SEM of 6-8 individual experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test. Significance (*) is defined as $p < 0.05$ when compared to the forskolin response alone.

8.3 Effect of the C-terminal Mutations on Melatonin-Mediated Inhibition of Forskolin-Induced cAMP Accumulation in COS-7 cells Expressing MT_1 and MT_2 Receptors that were Pretreated with 1 μM Melatonin

To determine if the C-terminal mutations affected the desensitization patterns of MT_1 and MT_2 receptors, COS-7 cells expressing either the WT or mutant forms of the receptors were first pretreated with 1 μM melatonin for 1 h, washed and then rechallenged with 10 nM melatonin for 20 min. It was expected that MT_1 WT, but not the MT_2 WT receptors would be resistant to desensitization as shown by previous studies (Witt-Enderby and Dubocovich, 1996; Witt-Enderby et al., 2003). It was expected that the mutations could alter the sensitivity state of MT_1 WT receptors by making them more prone to desensitization. Results show that pretreatment with melatonin desensitized MT_2 WT receptors reflected by attenuation in melatonin-induced inhibition of forskolin-induced cAMP accumulation. The selected mutations within the MT_2 receptors were without effect (Fig. 20). As for the MT_1 WT receptors, a 1 h pretreatment with melatonin did not desensitize the receptor reflected by no attenuation in melatonin-mediated inhibition of forskolin-induced cAMP accumulation. The C-terminal mutations were without effect on modifying the responses of the MT_1 or MT_2 receptors to this melatonin exposure.

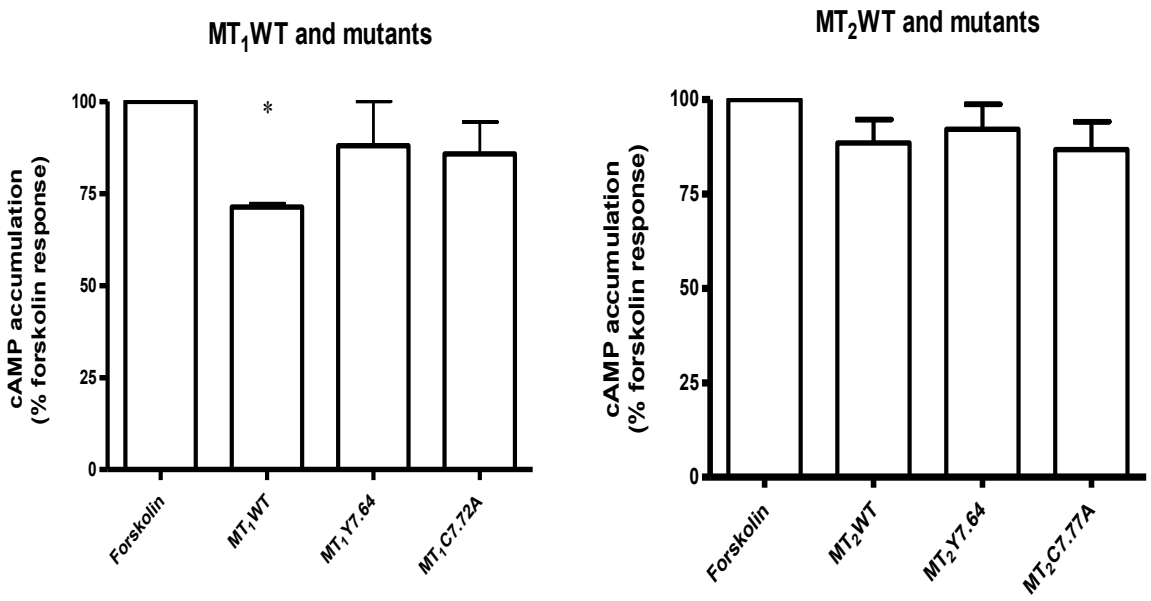


Figure 20: Melatonin Mediated Inhibition of Forskolin Induced cAMP Accumulation in Melatonin-Pretreated COS-7 cells Transiently Expressing WT or Mutant Melatonin Receptors

As shown, a rechallenge with 10 nM melatonin inhibited forskolin-induced cAMP accumulation in COS-7 cells expressing the wild type MT_1 receptor (MT_1 WT) even after a 1h prior exposure to melatonin. By contrast, no melatonin-mediated inhibition of forskolin-induced cAMP accumulation occurred in COS-7 cells expressing wild type MT_2 receptors (MT_2 WT). The C-terminal mutations for each of the melatonin receptor subtypes, MT_1 (MT_1C7.72A, MT_1Y7.64) or MT_2 (MT_2Y7.64 and MT_2C7.77A), were without effect when compared to WT receptors exposed to the same melatonin pretreatment. Each bar graph represents the mean ± SEM of 3-5 individual experiments performed in duplicate. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test. Significance (*) is defined as $p < 0.05$ when compared to forskolin response alone within each group.

8.4 Effect of the C-terminal Mutations on the Internalization Patterns of MT_1 and MT_2 Receptors Expressed in COS-7 Cells following Melatonin Exposure

All mutants and WT constructs used in this study were tagged with GFP, enabling visualization of the receptors through confocal microscopy to determine the location of the receptors in a cell. As shown in Fig. 21, the WT MT_1 and MT_2 receptors internalized following melatonin pretreatment, depicted by a punctate and cytosolic distribution of the GFP-tagged receptor (panels B and H) when compared with their vehicle controls (panels A and G). The cysteine mutants (MT_1C7.72A and MT_2C7.77A) also internalized in response to melatonin (panels D and J) when compared with their controls (panels C and I) similar to the WT receptors, indicating the lone cysteine residue in the C-terminal tails of these receptors did not play a role in receptor internalization. The truncation mutants (MT_1Y7.64 and MT_2Y7.64) did not internalize after melatonin treatment (panels F and L) when compared with their control cells treated with vehicle (E and K). The location of the GFP-tagged receptors remained at the plasma membrane even after melatonin exposure, suggesting that the C-terminal tail is essential for melatonin receptor internalization.

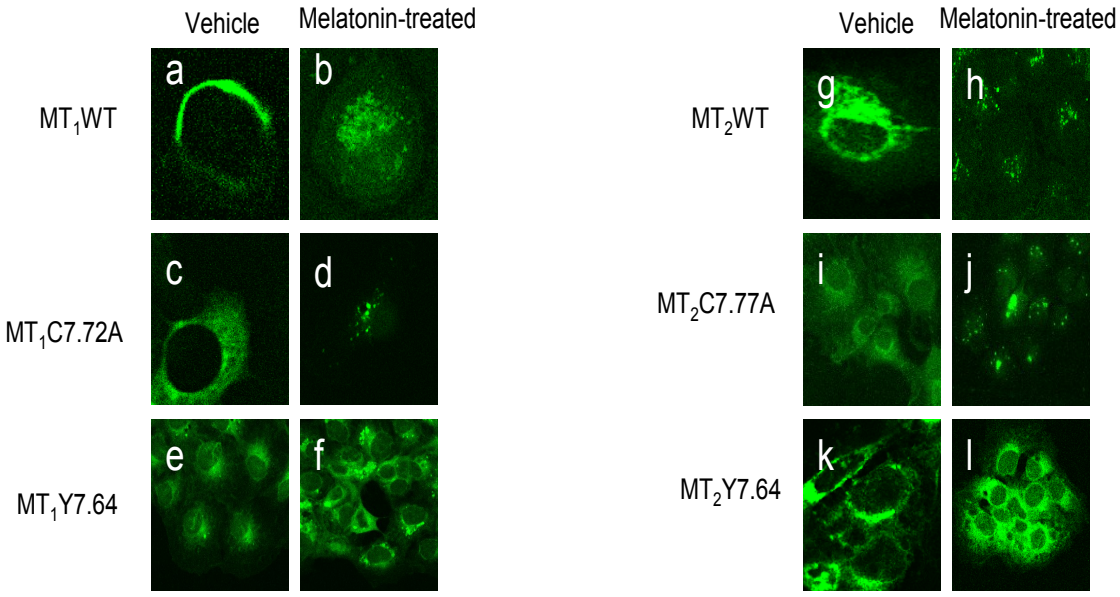


Figure 21: Internalization Patterns of Green Fluorescent Protein-Tagged WT and Mutant Receptors Following Exposure to Melatonin

As shown, a 1 h exposure to 1 μM melatonin (B and H) induced internalization of wild-type MT_1 and MT_2 receptors expressed in COS-7 cells when compared with vehicle controls (A and G). Mutation of the cysteine residue in each of the melatonin receptor subtypes, MT_1 (MT_1C7.72A) and MT_2 (MT_2C7.77A) receptors internalized in response to melatonin (D and J) similar to wild type (C and I). However, truncation of the C-terminal tail in each of the receptor subtypes, MT_1 (MT_1Y7.64) and MT_2 (MT_2Y7.64) prevented this internalization response to melatonin (F and I) when compared with their vehicle controls (E and K). Images are representative of six to seven independent experiments. Cells were visualized under oil immersion using a 100X-400X objective.

8.5 Effect of Palmitoylation on MT_1 WT and MT_2 WT Receptors.

To determine if the MT_1 WT and MT_2 WT melatonin receptors were palmitoylated COS cells transiently transfected with MT_1 WT and MT_2 WT melatonin receptors, were labeled in culture with 9,10-[^{3}H] palmitic acid for 16 h, and the incorporated radioactivity was measured. Palmitoylation at the C-terminal tail is thought to embed the tail in the cellular membrane, forming a 4th intracellular loop. As shown in Fig. 22, MT_1 WT, but not MT_2 WT receptors, incorporated significantly higher levels of [^{3}H] palmitic acid when compared with un-transfected COS-7 cells.

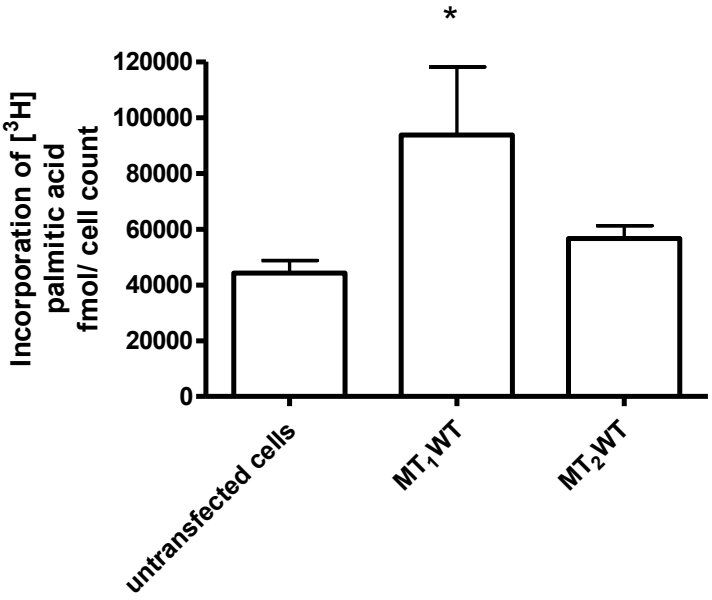


Figure 22: **Incorporation of 9, 10-[3H] palmitic acid in COS-7 Cells Transiently Transfected with MT_1 WT and MT_2 WT Receptors**

The incorporation of [3H] palmitic acid was significantly increased in MT_1WT constructs as compared to untransfected cells. Each bar graph represents the mean ± SEM of 3-4 individual experiments. The data was analyzed by one-way ANOVA followed by Newman-Keul's post hoc t- test. Significance (*) is defined as $P < 0.05$ when compared to untransfected cells.

9. DISCUSSION

The results from this study demonstrate the importance of the C-terminal domains within each of the MT_1 and MT_2 melatonin receptors as it relates to modulating receptor function and desensitization responses.

Essential to the study of receptor regulation using mutagenesis approaches is the requirement that the mutations do not affect the binding affinity of the agonist to its receptor. If the binding of the agonist to the receptor were to be affected because of the mutations, it would not be possible to determine if any change in receptor function was because of the change in binding or because of the mutations. As revealed through saturation binding analysis using the agonist radioligand, 2-[^{125}I]-iodomelatonin, there were no significant changes in the affinity (K_D) of 2-[^{125}I]-iodomelatonin for these mutated receptors compared to their respective WT receptors (Table 2) or to those values reported in the literature (Ekmekcioglu, 2006). It was expected that mutation of the C-terminal tails of each of the melatonin receptor subtypes would not affect agonist binding affinity because the ligand binding site for melatonin receptors is thought to lie between TM 5-7 (Navajas et al., 1996; Barrett et al., 2003), and TM 3 (Navajas et al., 1996; Kokkola et al., 2003) for MT_1 receptors and TM 4-5 for MT_2 receptors (Barrett et al., 2003; Gerdin et al., 2003b).

In the first type of mutation, the cysteine residue in the C-terminal tail of each receptor was mutated to alanine (MT_1C7.72A and MT_2C7.77A), which does not contain the thiol groups capable of being palmitoylated, and therefore, was an ideal choice for the mutation. This cysteine is a palmitoylation site in many other GPCRs, and serves to form a fourth intracellular loop by embedding the palmitoyl groups into the membrane. This

loop is important in G-protein binding and activation of the rhodopsin receptor (Ernst et al., 2000), and the absence of this loop in some receptors has led to G protein uncoupling (O'Dowd et al., 1989a; Hayashi and Haga, 1997). In this study, mutation of the cysteine residue within the C-terminal domain of each of the melatonin receptor subtypes, MT_1 and MT_2 (MT_1C7.72A and MT_2C7.77A), blocked the transfer of signal from the receptor to inhibit cAMP production. The absence of this potential palmitoylation site within each of the melatonin receptor subtypes possibly disrupts the formation of the fourth intracellular loop; a loop that may bind directly to G_i-proteins. This disruption in receptor/ G_i-protein coupling may result in attenuated cAMP inhibitory responses coming from their respective receptors.

Uncoupling of melatonin receptors from G_i proteins using either GTPγS or pertussis toxin results in attenuated cAMP inhibitory responses in CHO cells (Witt-Enderby and Dubocovich, 1996). Though not tested in this study, it is also possible that the cysteine mutations make the melatonin receptors more prone to desensitization by somehow allowing easier access of G-protein receptor kinases to these phosphorylation sites. This hypothesis of heightened sensitization is speculative and more studies are required to support this idea. Similar results have been reported for β_2-adrenergic receptor, where in addition to being uncoupled from G_α, a palmitoylation mutant (Cys341Gly) was hyperphosphorylated (Moffett et al., 1993). Although there may be additional residues in the C-terminal tail in addition to these cysteine residues, which are required for downstream cAMP signaling of the MT_1 and MT_2 receptors, taken together, the findings from both C-terminal mutations suggest that cysteine 7.72 in MT_1 receptors

and 7.77 in MT_2 receptors plays an important role in transducing cAMP responses throughout the cell.

Desensitization is a process in which a receptor becomes refractory to its agonist over time. Many reports have shown that melatonin receptors become desensitized following exposure to melatonin either directly (Gerdin et al., 2003a; Jarzynka MJ et al., 2006; Bondi et al., 2007) or during the night when melatonin levels are highest (Gauer et al., 1994). In this study, MT_2 WT receptors but not MT_1 receptors desensitized after a 1h pretreatment with 1 μM melatonin. These data are consistent with previous studies (Witt-Enderby and Dubocovich, 1996; Witt-Enderby et al., 2003). In this study, neither of the C-terminal mutations altered the responses of each of the receptor subtypes (MT_1, MT_2) to a 1h exposure to 1 μM melatonin. These data suggest that the lack of signal transfer through each of the melatonin receptors due to the cysteine 7.72 (MT_1) or 7.77 (MT_2) mutation prevented these receptor mutants from responding to the melatonin exposure like their WT counterparts.

Besides desensitizing through uncoupling, melatonin receptors, in response to agonist exposure, also desensitize by internalization of the receptors (Gerdin et al., 2003a; Gerdin et al., 2003b; Bondi et al., 2007). As revealed through the internalization assays, truncation of the C-tail of each of the melatonin receptor subtypes (MT_1Y7.64 or MT_2Y7.64) prevented melatonin-induced internalization of MT_1 and MT_2 receptors when compared to their WT counterparts. By contrast, the cysteine point mutations at position 7.72 for MT_1 receptors or position 7.77 for MT_2 receptors displayed internalization patterns similar to what was observed for their WT receptors. As described earlier, blocking the palmitoylation reduced the number of receptors reaching the cell surface in

TSH receptors, δ-opioid receptors and histamine H2 receptors (Tanaka et al., 1998; Fukushima et al., 2001; Petaja-Repo et al., 2006). The internalization data from the cysteine mutations show that unlike these receptors, the cysteine mutated receptors were able to traffic normally to the cell surface, similar to the WT receptors. These data suggest that these amino acids are not involved in melatonin-induced internalization processes.

Overall, the findings from the internalization assays show that the C-terminal tails within the MT_1 and MT_2 WT receptors function in melatonin receptor internalization. As such, removal of these sites through truncation mutagenesis prevented melatonin-induced internalization responses. Because these C-terminal tails contain many putative phosphorylation sites, these tails may modulate melatonin receptor internalization by providing the binding site to which beta-arrestins can bind similar to what occurs for other GPCRs (Ferguson, 2001).

Palmitoylation assays demonstrate that the MT_1 WT receptors are palmitoylated when compared to non-transfected COS-7 cells. The palmitoylation of MT_2 WT receptors was not significantly different than untransfected cells, which may suggest that palmitoylation occurs in MT_1 receptors (perhaps in the C-tail) but not in MT_2 receptors. Even though no significant difference in [^{3}H] palmitate incorporation occurred in MT_2 receptors, this could be due to a limitation to the assay conditions (i.e., transiently transfected cells). More experiments need to be performed in this area to support or refute this idea.

10. CONCLUSION

This study reveals many important findings regarding those domains within the melatonin receptor that underlie desensitization processes. Considering that desensitization events may underlie melatonin-induced cellular differentiation (Radio et al., 2006; Bondi et al., 2007), then understanding the role that specific domains play in melatonin receptor function may lead to novel melatonin therapies targeted to enhance cellular differentiation such as for the treatment of cancer and osteoporosis (Witt-Enderby et al., 2006). Additionally, the knowledge gained from this study could lead to novel therapeutic targets as it relates to melatonin and melatonin-related disorders including insomnia and depression.

11. REFERENCES

http://www.nof.org/osteoporosis/diseasefacts.htm. *National Osteoporosis Foundation.*:Accessed October 20, 2009.

Acuna-Castroviejo D, Escames G, Rodriguez MI and Lopez LC (2007) Melatonin role in the mitochondrial function. *Front Biosci* **12:**947-963.

Adam-Vizi V and Chinopoulos C (2006) Bioenergetics and the formation of mitochondrial reactive oxygen species. *Trends Pharmacol Sci* **27:**639-645.

Ahmed I, Gesty-Palmer D, Drezner MK and Luttrell LM (2003) Transactivation of the epidermal growth factor receptor mediates parathyroid hormone and prostaglandin F2 alpha-stimulated mitogen-activated protein kinase activation in cultured transgenic murine osteoblasts. *Mol Endocrinol* **17:**1607-1621.

Alarma-Estrany P and Pintor J (2007) Melatonin receptors in the eye: location, second messengers and role in ocular physiology. *Pharmacol Ther* **113:**507-522.

Arney KL and Fisher AG (2004) Epigenetic aspects of differentiation. *J Cell Sci* **117:**4355-4363.

Arthur A, Zannettino A and Gronthos S (2009) The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. *J Cell Physiol* **218:**237-245.

Ballesteros J and Weinstein. H (1995) Integrated methods for the construction of three dimensional models of structure-function relations in G-protein coupled receptors. *Methods Neurosci* **25:**366-428.

Bancroft GN, Sikavitsas VI, van den Dolder J, Sheffield TL, Ambrose CG, Jansen JA and Mikos AG (2002) Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc Natl Acad Sci U S A* **99:**12600-12605.

Banfi G, Iorio EL and Corsi MM (2008) Oxidative stress, free radicals and bone remodeling. *Clin Chem Lab Med* **46:**1550-1555.

Barrett P, Conway S and Morgan PJ (2003) Digging deep--structure-function relationships in the melatonin receptor family. *J Pineal Res* **35:**221-230.

Benitez-King G, Huerto-Delgadillo L and Anton-Tay F (1993) Binding of 3H-melatonin to calmodulin. *Life Sci* **53:**201-207.

Berger CE, Horrocks BR and Datta HK (1999) Direct non-genomic effect of steroid hormones on superoxide anion generation in the bone resorbing osteoclasts. *Mol Cell Endocrinol* **149:**53-59.

Bondi CD, McKeon RM, Bennett JM, Ignatius PF, Brydon L, Jockers R, Melan MA and Witt-Enderby PA (2008) MT1 melatonin receptor internalization underlies melatonin-induced morphologic changes in Chinese hamster ovary cells and these processes are dependent on Gi proteins, MEK 1/2 and microtubule modulation. *J Pineal Res* **44:**288-298.

Boyce BF and Xing L (2007) The RANKL/RANK/OPG pathway. *Curr Osteoporos Rep* **5:**98-104.

Brixen KT, Christensen PM, Ejersted C and Langdahl BL (2004) Teriparatide (biosynthetic human parathyroid hormone 1-34): a new paradigm in the treatment of osteoporosis. *Basic Clin Pharmacol Toxicol* **94:**260-270.

Bruder SP, Jaiswal N and Haynesworth SE (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* **64:**278-294.

Brydon L, Roka F, Petit L, de Coppet P, Tissot M, Barrett P, Morgan PJ, Nanoff C, Strosberg AD and Jockers R (1999) Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. *Mol Endocrinol* **13:**2025-2038.

Burgering BM and Bos JL (1995) Regulation of Ras-mediated signalling: more than one way to skin a cat. *Trends Biochem Sci* **20:**18-22.

Cai Z, Kwintkiewicz J, Young ME and Stocco C (2007) Prostaglandin E2 increases cyp19 expression in rat granulosa cells: implication of GATA-4. *Mol Cell Endocrinol* **263:**181-189.

Cardinali DP, Ladizesky MG, Boggio V, Cutrera RA and Mautalen C (2003) Melatonin effects on bone: experimental facts and clinical perspectives. *J Pineal Res* **34:**81-87.

Carlson LL, Weaver DR and Reppert SM (1989) Melatonin signal transduction in hamster brain: inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein. *Endocrinology* **125:**2670-2676.

Chansard M, Iwahana E, Liang J and Fukuhara C (2005) Regulation of cAMP-induced arylalkylamine N-acetyltransferase, Period1, and MKP-1 gene expression by mitogen-activated protein kinases in the rat pineal gland. *Brain Res Mol Brain Res* **139:**333-340.

Chau JF, Leong WF and Li B (2009) Signaling pathways governing osteoblast proliferation, differentiation and function. *Histol Histopathol* **24:**1593-1606.

Chen CT, Shih YR, Kuo TK, Lee OK and Wei YH (2008a) Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. *Stem Cells* **26:**960-968.

Chen D, Zhao M and Mundy GR (2004) Bone morphogenetic proteins. *Growth Factors* **22:**233-241.

Chen H, Shoumura S, Emura S, Utsumi M, Yamahira T and Isono H (1990) Effects of pinealectomy on the ultrastructure of the golden hamster parathyroid gland. *Histol Histopathol* **5:**477-484.

Chen JR, Shankar K, Nagarajan S, Badger TM and Ronis MJ (2008b) Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and downstream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-kappaB ligand signaling cascade. *J Pharmacol Exp Ther* **324:**50-59.

Cheng SL, Yang JW, Rifas L, Zhang SF and Avioli LV (1994) Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *Endocrinology* **134:**277-286.

Cheung KM, Wang T, Poon AM, Carl A, Tranmer B, Hu Y, Luk KD and Leong JC (2005) The effect of pinealectomy on scoliosis development in young nonhuman primates. *Spine (Phila Pa 1976)* **30:**2009-2013.

Chini B and Parenti M (2009) G-protein-coupled receptors, cholesterol and palmitoylation: facts about fats. *J Mol Endocrinol* **42:**371-379.

Cho YM, Kwon S, Pak YK, Seol HW, Choi YM, Park do J, Park KS and Lee HK (2006) Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun* **348:**1472-1478.

Christenson RH (1997) Biochemical markers of bone metabolism: an overview. *Clin Biochem* **30:**573-593.

Christiansen C (1993) Skeletal osteoporosis. *J Bone Miner Res* **8 Suppl 2:**S475-480.

Conti A, Conconi S, Hertens E, Skwarlo-Sonta K, Markowska M and Maestroni JM (2000) Evidence for melatonin synthesis in mouse and human bone marrow cells. *J Pineal Res* **28:**193-202.

Dai J, Inscho EW, Yuan L and Hill SM (2002) Modulation of intracellular calcium and calmodulin by melatonin in MCF-7 human breast cancer cells. *J Pineal Res* **32:**112-119.

Das S, Ise T, Nagata S, Maeda H, Bera TK and Pastan I (2007) Palmitoylation of POTE family proteins for plasma membrane targeting. *Biochem Biophys Res Commun* **363:**751-756.

Day TF, Guo X, Garrett-Beal L and Yang Y (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell* **8:**739-750.

Delagrange P, Atkinson J, Boutin JA, Casteilla L, Lesieur D, Misslin R, Pellissier S, Penicaud L and Renard P (2003) Therapeutic perspectives for melatonin agonists and antagonists. *J Neuroendocrinol* **15:**442-448.

Delmas PD (2002) Treatment of postmenopausal osteoporosis. *Lancet* **359:**2018-2026.

Derynck R and Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425:**577-584.

DeWire SM, Ahn S, Lefkowitz RJ and Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* **69:**483-510.

Dotto GP (1999) Signal transduction pathways controlling the switch between keratinocyte growth and differentiation. *Crit Rev Oral Biol Med* **10:**442-457.

Drew JE, Barrett P, Williams LM, Conway S and Morgan PJ (1998) The ovine melatonin-related receptor: cloning and preliminary distribution and binding studies. *J Neuroendocrinol* **10:**651-661.

Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ and Masana MI (2003) Molecular pharmacology, regulation and function of mammalian melatonin receptors. *Front Biosci* **8:**d1093-1108.

Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M and Karsenty G (1999) A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev* **13:**1025-1036.

Ducy P, Zhang R, Geoffroy V, Ridall AL and Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89:**747-754.

Duguez S, Feasson L, Denis C and Freyssenet D (2002) Mitochondrial biogenesis during skeletal muscle regeneration. *Am J Physiol Endocrinol Metab* **282:**E802-809.

Eijken M, Hewison M, Cooper MS, de Jong FH, Chiba H, Stewart PM, Uitterlinden AG, Pols HA and van Leeuwen JP (2005) 11beta-Hydroxysteroid dehydrogenase expression and glucocorticoid synthesis are directed by a molecular switch during osteoblast differentiation. *Mol Endocrinol* **19:**621-631.

Ekmekcioglu C (2006) Melatonin receptors in humans: biological role and clinical relevance. *Biomed Pharmacother* **60:**97-108.

Engin F, Yao Z, Yang T, Zhou G, Bertin T, Jiang MM, Chen Y, Wang L, Zheng H, Sutton RE, Boyce BF and Lee B (2008) Dimorphic effects of Notch signaling in bone homeostasis. *Nat Med* **14:**299-305.

Ernst OP, Meyer CK, Marin EP, Henklein P, Fu WY, Sakmar TP and Hofmann KP (2000) Mutation of the fourth cytoplasmic loop of rhodopsin affects binding of transducin

and peptides derived from the carboxyl-terminal sequences of transducin alpha and gamma subunits. *J Biol Chem* **275:**1937-1943.

Facucho-Oliveira JM and St John JC (2009) The relationship between pluripotency and mitochondrial DNA proliferation during early embryo development and embryonic stem cell differentiation. *Stem Cell Rev* **5:**140-158.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53:**1-24.

Feskanich D, Hankinson SE and Schernhammer ES (2009) Nightshift work and fracture risk: the Nurses' Health Study. *Osteoporos Int* **20:**537-542.

Fjelldal PG, Grotmol S, Kryvi H, Gjerdet NR, Taranger GL, Hansen T, Porter MJ and Totland GK (2004) Pinealectomy induces malformation of the spine and reduces the mechanical strength of the vertebrae in Atlantic salmon, Salmo salar. *J Pineal Res* **36:**132-139.

Franceschi RT, Ge C, Xiao G, Roca H and Jiang D (2007) Transcriptional regulation of osteoblasts. *Ann N Y Acad Sci* **1116:**196-207.

Fraser JH, Helfrich MH, Wallace HM and Ralston SH (1996) Hydrogen peroxide, but not superoxide, stimulates bone resorption in mouse calvariae. *Bone* **19:**223-226.

Fukushima Y, Saitoh T, Anai M, Ogihara T, Inukai K, Funaki M, Sakoda H, Onishi Y, Ono H, Fujishiro M, Ishikawa T, Takata K, Nagai R, Omata M and Asano T (2001) Palmitoylation of the canine histamine H2 receptor occurs at Cys(305) and is important for cell surface targeting. *Biochim Biophys Acta* **1539:**181-191.

Gao Z, Ni Y, Szabo G and Linden J (1999) Palmitoylation of the recombinant human A1 adenosine receptor: enhanced proteolysis of palmitoylation-deficient mutant receptors. *Biochem J* **342 (Pt 2):**387-395.

Gauer F, Masson-Pevet M, Stehle J and Pevet P (1994) Daily variations in melatonin receptor density of rat pars tuberalis and suprachiasmatic nuclei are distinctly regulated. *Brain Res* **641:**92-98.

Ge C, Xiao G, Jiang D and Franceschi RT (2007) Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol* **176:**709-718.

Gerdin MJ, Masana MI, Ren D, Miller RJ and Dubocovich ML (2003a) Short-term exposure to melatonin differentially affects the functional sensitivity and trafficking of the hMT1 and hMT2 melatonin receptors. *J Pharmacol Exp Ther* **304:**931-939.

Gerdin MJ, Mseeh F and Dubocovich ML (2003b) Mutagenesis studies of the human MT2 melatonin receptor. *Biochem Pharmacol* **66:**315-320.

Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, Eckhardt AE, Cowan CL, Spurney RF, Luttrell LM and Lefkowitz RJ (2006) Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* **281:**10856-10864.

Graham C, Cook MR, Gerkovich MM and Sastre A (2001) Examination of the melatonin hypothesis in women exposed at night to EMF or bright light. *Environ Health Perspect* **109:**501-507.

Grant SG, Melan MA, Latimer JJ and Witt-Enderby PA (2009) Melatonin and breast cancer: cellular mechanisms, clinical studies and future perspectives. *Expert Rev Mol Med* **11:**e5.

Greenspan SL, Dresner-Pollak R, Parker RA, London D and Ferguson L (1997) Diurnal variation of bone mineral turnover in elderly men and women. *Calcif Tissue Int* **60:**419-423.

Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G and Caverzasio J (2003) Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. *J Bone Miner Res* **18:**2060-2068.

Guo X and Wang XF (2009) Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res* **19:**71-88.

Hardeland R (2009) Melatonin: signaling mechanisms of a pleiotropic agent. *Biofactors* **35:**183-192.

Hardeland R and Pandi-Perumal SR (2005) Melatonin, a potent agent in antioxidative defense: actions as a natural food constituent, gastrointestinal factor, drug and prodrug. *Nutr Metab (Lond)* **2:**22.

Hassager C, Risteli J, Risteli L, Jensen SB and Christiansen C (1992) Diurnal variation in serum markers of type I collagen synthesis and degradation in healthy premenopausal women. *J Bone Miner Res* **7:**1307-1311.

Hayashi MK and Haga T (1997) Palmitoylation of muscarinic acetylcholine receptor m2 subtypes: reduction in their ability to activate G proteins by mutation of a putative palmitoylation site, cysteine 457, in the carboxyl-terminal tail. *Arch Biochem Biophys* **340:**376-382.

Heshmati HM, Riggs BL, Burritt MF, McAlister CA, Wollan PC and Khosla S (1998) Effects of the circadian variation in serum cortisol on markers of bone turnover and calcium homeostasis in normal postmenopausal women. *J Clin Endocrinol Metab* **83:**751-756.

Hickman AB, Klein DC and Dyda F (1999) Melatonin biosynthesis: the structure of serotonin N-acetyltransferase at 2.5 A resolution suggests a catalytic mechanism. *Mol Cell* **3:**23-32.

Hock JM and Gera I (1992) Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone. *J Bone Miner Res* **7:**65-72.

Horsman A, Gallagher JC, Simpson M and Nordin BE (1977) Prospective trial of oestrogen and calcium in postmenopausal women. *Br Med J* **2:**789-792.

Huang NF and Li S (2008) Mesenchymal stem cells for vascular regeneration. *Regen Med* **3:**877-892.

Huang W, Yang S, Shao J and Li YP (2007) Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front Biosci* **12:**3068-3092.

Hukovic N, Panetta R, Kumar U, Rocheville M and Patel YC (1998) The cytoplasmic tail of the human somatostatin receptor type 5 is crucial for interaction with adenylyl cyclase and in mediating desensitization and internalization. *J Biol Chem* **273:**21416-21422.

Ishida Y and Kawai S (2004) Comparative efficacy of hormone replacement therapy, etidronate, calcitonin, alfacalcidol, and vitamin K in postmenopausal women with osteoporosis: The Yamaguchi Osteoporosis Prevention Study. *Am J Med* **117:**549-555.

Jaiswal N, Haynesworth SE, Caplan AI and Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem* **64:**295-312.

Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR and Pittenger MF (2000) Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J Biol Chem* **275:**9645-9652.

Jarzynka MJ, Passey DK, Ignatius PF, Melan MA, Radio NM, R J, MM R and L B (2006) Modulation of melatonin receptors and G-protein function by microtubules. *J Pineal Res* **41:**324-336.

Jin H, Zastawny R, George SR and O'Dowd BF (1997) Elimination of palmitoylation sites in the human dopamine D1 receptor does not affect receptor-G protein interaction. *Eur J Pharmacol* **324:**109-116.

Jorgensen NR, Henriksen Z, Sorensen OH and Civitelli R (2004) Dexamethasone, BMP-2, and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an in vitro model for human bone marrow-derived primary osteoblasts. *Steroids* **69:**219-226.

Karp JM and Leng Teo GS (2009) Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* **4:**206-216.

Kennedy ME and Limbird LE (1994) Palmitoylation of the alpha 2A-adrenergic receptor. Analysis of the sequence requirements for and the dynamic properties of alpha 2A-adrenergic receptor palmitoylation. *J Biol Chem* **269:**31915-31922.

Kim SH, Kim YH, Song M, An SH, Byun HY, Heo K, Lim S, Oh YS, Ryu SH and Suh PG (2007) O-GlcNAc modification modulates the expression of osteocalcin via OSE2 and Runx2. *Biochem Biophys Res Commun* **362:**325-329.

Kokkola T, Foord SM, Watson MA, Vakkuri O and Laitinen JT (2003) Important amino acids for the function of the human MT1 melatonin receptor. *Biochem Pharmacol* **65:**1463-1471.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S and Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89:**755-764.

Korf HW, Schomerus C and Stehle JH (1998) The pineal organ, its hormone melatonin, and the photoneuroendocrine system. *Adv Anat Embryol Cell Biol* **146:**1-100.

Korkmaz A, Sanchez-Barcelo EJ, Tan DX and Reiter RJ (2009) Role of melatonin in the epigenetic regulation of breast cancer. *Breast Cancer Res Treat* **115:**13-27.

Kovacs JJ, Hara MR, Davenport CL, Kim J and Lefkowitz RJ (2009) Arrestin development: emerging roles for beta-arrestins in developmental signaling pathways. *Dev Cell* **17:**443-458.

Koyama H, Nakade O, Takada Y, Kaku T and Lau KH (2002) Melatonin at pharmacologic doses increases bone mass by suppressing resorption through down-regulation of the RANKL-mediated osteoclast formation and activation. *J Bone Miner Res* **17:**1219-1229.

Kwok S, Partridge NC, Srinivasan N, Nair SV and Selvamurugan N (2009) Mitogen activated protein kinase-dependent inhibition of osteocalcin gene expression by transforming growth factor-beta1. *J Cell Biochem* **106:**161-169.

Ladizesky MG, Cutrera RA, Boggio V, Somoza J, Centrella JM, Mautalen C and Cardinali DP (2001) Effect of melatonin on bone metabolism in ovariectomized rats. *Life Sci* **70:**557-565.

Lai CF and Cheng SL (2002) Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. *J Biol Chem* **277:**15514-15522.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM and Bae SC (2000) Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5

induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol* **20:**8783-8792.

Lee MH, Kim YJ, Kim HJ, Park HD, Kang AR, Kyung HM, Sung JH, Wozney JM and Ryoo HM (2003) BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. *J Biol Chem* **278:**34387-34394.

Lian JB, Stein GS, Javed A, van Wijnen AJ, Stein JL, Montecino M, Hassan MQ, Gaur T, Lengner CJ and Young DW (2006) Networks and hubs for the transcriptional control of osteoblastogenesis. *Rev Endocr Metab Disord* **7:**1-16.

Lincoln G, Messager S, Andersson H and Hazlerigg D (2002) Temporal expression of seven clock genes in the suprachiasmatic nucleus and the pars tuberalis of the sheep: evidence for an internal coincidence timer. *Proc Natl Acad Sci U S A* **99:**13890-13895.

Lindsay R, Hart DM, Aitken JM, MacDonald EB, Anderson JB and Clarke AC (1976) Long-term prevention of postmenopausal osteoporosis by oestrogen. Evidence for an increased bone mass after delayed onset of oestrogen treatment. *Lancet* **1:**1038-1041.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:**402-408.

Locklin RM, Khosla S, Turner RT and Riggs BL (2003) Mediators of the biphasic responses of bone to intermittent and continuously administered parathyroid hormone. *J Cell Biochem* **89:**180-190.

Lowell S, Benchoua A, Heavey B and Smith AG (2006) Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol* **4:**e121.

Lufkin EG and Ory SJ (1989) Estrogen replacement therapy for the prevention of osteoporosis. *Am Fam Physician* **40:**205-212.

Machida M, Dubousset J, Imamura Y, Iwaya T, Yamada T and Kimura J (1993) An experimental study in chickens for the pathogenesis of idiopathic scoliosis. *Spine (Phila Pa 1976)* **18:**1609-1615.

Machida M, Dubousset J, Yamada T and Kimura J (2009) Serum melatonin levels in adolescent idiopathic scoliosis prediction and prevention for curve progression--a prospective study. *J Pineal Res* **46:**344-348.

MacKenzie RS, Melan MA, Passey DK and Witt-Enderby PA (2002) Dual coupling of MT(1) and MT(2) melatonin receptors to cyclic AMP and phosphoinositide signal transduction cascades and their regulation following melatonin exposure. *Biochem Pharmacol* **63:**587-595.

Manolagas SC (2000) Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev* **21:**115-137.

Martinez-Campa C, Alonso-Gonzalez C, Mediavilla MD, Cos S, Gonzalez A, Ramos S and Sanchez-Barcelo EJ (2006) Melatonin inhibits both ER alpha activation and breast cancer cell proliferation induced by a metalloestrogen, cadmium. *J Pineal Res* **40:**291-296.

Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, Aburatani H, Nishimura R and Yoneda T (2008) BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* **283:**29119-29125.

Matsumoto T, Igarashi C, Takeuchi Y, Harada S, Kikuchi T, Yamato H and Ogata E (1991) Stimulation by 1,25-dihydroxyvitamin D3 of in vitro mineralization induced by osteoblast-like MC3T3-E1 cells. *Bone* **12:**27-32.

Matsushita T, Chan YY, Kawanami A, Balmes G, Landreth GE and Murakami S (2009) Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis. *Mol Cell Biol* **29:**5843-5857.

Mazna P, Berka K, Jelinkova I, Balik A, Svoboda P, Obsilova V, Obsil T and Teisinger J (2005) Ligand binding to the human MT2 melatonin receptor: the role of residues in transmembrane domains 3, 6, and 7. *Biochem Biophys Res Commun* **332:**726-734.

Mazna P, Obsilova V, Jelinkova I, Balik A, Berka K, Sovova Z, Ettrich R, Svoboda P, Obsil T and Teisinger J (2004) Molecular modeling of human MT2 melatonin receptor: the role of Val204, Leu272 and Tyr298 in ligand binding. *J Neurochem* **91:**836-842.

McNulty S, Ross AW, Barrett P, Hastings MH and Morgan PJ (1994) Melatonin regulates the phosphorylation of CREB in ovine pars tuberalis. *J Neuroendocrinol* **6:**523-532.

Miyazono K, Maeda S and Imamura T (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev* **16:**251-263.

Moench SJ, Moreland J, Stewart DH and Dewey TG (1994) Fluorescence studies of the location and membrane accessibility of the palmitoylation sites of rhodopsin. *Biochemistry* **33:**5791-5796.

Moffett S, Mouillac B, Bonin H and Bouvier M (1993) Altered phosphorylation and desensitization patterns of a human beta 2-adrenergic receptor lacking the palmitoylated Cys341. *EMBO J* **12:**349-356.

Molis TM, Spriggs LL and Hill SM (1994) Modulation of estrogen receptor mRNA expression by melatonin in MCF-7 human breast cancer cells. *Mol Endocrinol* **8:**1681-1690.

Moreau A, Wang DS, Forget S, Azeddine B, Angeloni D, Fraschini F, Labelle H, Poitras B, Rivard CH and Grimard G (2004) Melatonin signaling dysfunction in adolescent idiopathic scoliosis. *Spine (Phila Pa 1976)* **29:**1772-1781.

Munshi UM, Peegel H and Menon KM (2001) Palmitoylation of the luteinizing hormone/human chorionic gonadotropin receptor regulates receptor interaction with the arrestin-mediated internalization pathway. *Eur J Biochem* **268:**1631-1639.

Nakade O, Koyama H, Ariji H, Yajima A and Kaku T (1999) Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro. *J Pineal Res* **27:**106-110.

Nakamura A, Dohi Y, Akahane M, Ohgushi H, Nakajima H, Funaoka H and Takakura Y (2009) Osteocalcin secretion as an early marker of in vitro osteogenic differentiation of rat mesenchymal stem cells. *Tissue Eng Part C Methods* **15:**169-180.

Nakashima A and Tamura M (2006) Regulation of matrix metalloproteinase-13 and tissue inhibitor of matrix metalloproteinase-1 gene expression by WNT3A and bone morphogenetic protein-2 in osteoblastic differentiation. *Front Biosci* **11:**1667-1678.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR and de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108:**17-29.

Navajas C, Kokkola T, Poso A, Honka N, Gynther J and Laitinen JT (1996) A rhodopsin-based model for melatonin recognition at its G protein-coupled receptor. *Eur J Pharmacol* **304:**173-183.

Nelson DR KT, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, et al. (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12:**1-51.

Nosjean O, Ferro M, Coge F, Beauverger P, Henlin JM, Lefoulon F, Fauchere JL, Delagrange P, Canet E and Boutin JA (2000) Identification of the melatonin-binding site MT3 as the quinone reductase 2. *J Biol Chem* **275:**31311-31317.

Nosjean O, Nicolas JP, Klupsch F, Delagrange P, Canet E and Boutin JA (2001) Comparative pharmacological studies of melatonin receptors: MT1, MT2 and MT3/QR2. Tissue distribution of MT3/QR2. *Biochem Pharmacol* **61:**1369-1379.

O'Dowd B, Hnatowich M, Caron MG, Lefkowitz RJ and Bouvier M (1989a) Palmitoylation of the human ß2-adrenergic receptor. *J. Biol. Chem***:**7564.

O'Dowd BF, Hnatowich M, Caron MG, Lefkowitz RJ and Bouvier M (1989b) Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J Biol Chem* **264:**7564-7569.

Ostrowska Z, Kos-Kudla B, Marek B and Kajdaniuk D (2003) Influence of lighting conditions on daily rhythm of bone metabolism in rats and possible involvement of melatonin and other hormones in this process. *Endocr Regul* **37:**163-174.

Ostrowska Z, Kos-Kudla B, Marek B, Swietochowska E and Gorski J (2001a) Assessment of the relationship between circadian variations of salivary melatonin levels and type I collagen metabolism in postmenopausal obese women. *Neuro Endocrinol Lett* **22:**121-127.

Ostrowska Z, Kos-Kudla B, Swietochowska E, Marek B, Kajdaniuk D and Gorski J (2001b) Assessment of the relationship between dynamic pattern of nighttime levels of melatonin and chosen biochemical markers of bone metabolism in a rat model of postmenopausal osteoporosis. *Neuro Endocrinol Lett* **22:**129-136.

Otsuka E, Yamaguchi A, Hirose S and Hagiwara H (1999) Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Am J Physiol* **277:**C132-138.

Ovchinnikov Yu A, Abdulaev NG and Bogachuk AS (1988) Two adjacent cysteine residues in the C-terminal cytoplasmic fragment of bovine rhodopsin are palmitylated. *FEBS Lett* **230:**1-5.

Oyama J, Murai I, Kanazawa K and Machida M (2006) Bipedal ambulation induces experimental scoliosis in C57BL/6J mice with reduced plasma and pineal melatonin levels. *J Pineal Res* **40:**219-224.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289:**739-745.

Petaja-Repo UE, Hogue M, Leskela TT, Markkanen PM, Tuusa JT and Bouvier M (2006) Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human delta opioid receptor. *J Biol Chem* **281:**15780-15789.

Petit L, Lacroix I, de Coppet P, Strosberg AD and Jockers R (1999) Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3'-5'-monophosphate pathway. *Biochem Pharmacol* **58:**633-639.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284:**143-147.

Premont RT and Gainetdinov RR (2007) Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu Rev Physiol* **69:**511-534.

Qanbar R and Bouvier M (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacol Ther* **97:**1-33.

Radio NM, Doctor JS and Witt-Enderby PA (2006) Melatonin enhances alkaline phosphatase activity in differentiating human adult mesenchymal stem cells grown in osteogenic medium via MT2 melatonin receptors and the MEK/ERK (1/2) signaling cascade. *J Pineal Res* **40:**332-342.

Rato AG, Pedrero JG, Martinez MA, del Rio B, Lazo PS and Ramos S (1999) Melatonin blocks the activation of estrogen receptor for DNA binding. *FASEB J* **13:**857-868.

Rawadi G, Ferrer C, Spinella-Jaegle S, Roman-Roman S, Bouali Y and Baron R (2001) 1-(5-oxohexyl)-3,7-Dimethylxanthine, a phosphodiesterase inhibitor, activates MAPK cascades and promotes osteoblast differentiation by a mechanism independent of PKA activation (pentoxifylline promotes osteoblast differentiation). *Endocrinology* **142:**4673-4682.

Rawadi G, Vayssiere B, Dunn F, Baron R and Roman-Roman S (2003) BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J Bone Miner Res* **18:**1842-1853.

Reiter RJ and Tan DX (2003) What constitutes a physiological concentration of melatonin? *J Pineal Res* **34:**79-80.

Reiter RJ, Tan DX and Allegra M (2002) Melatonin: reducing molecular pathology and dysfunction due to free radicals and associated reactants. *Neuro Endocrinol Lett* **23 Suppl 1:**3-8.

Reiter RJ, Tan DX, Manchester LC, Pilar Terron M, Flores LJ and Koppisepi S (2007a) Medical implications of melatonin: receptor-mediated and receptor-independent actions. *Adv Med Sci* **52:**11-28.

Reiter RJ, Tan DX, Manchester LC and Qi W (2001) Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. *Cell Biochem Biophys* **34:**237-256.

Reiter RJ, Tan DX, Mayo JC, Sainz RM, Leon J and Czarnocki Z (2003) Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochim Pol* **50:**1129-1146.

Reiter RJ, Tan DX, Terron MP, Flores LJ and Czarnocki Z (2007b) Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. *Acta Biochim Pol* **54:**1-9.

Reppert SM, Godson C, Mahle CD, Weaver DR, Slaugenhaupt SA and Gusella JF (1995) Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. *Proc Natl Acad Sci U S A* **92:**8734-8738.

Reppert SM and Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* **418:**935-941.

Reppert SM, Weaver DR and Ebisawa T (1994) Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* **13:**1177-1185.

Reppert SM, Weaver DR, Ebisawa T, Mahle CD and Kolakowski LF, Jr. (1996) Cloning of a melatonin-related receptor from human pituitary. *FEBS Lett* **386:**219-224.

Robinson LJ, Yaroslavskiy BB, Griswold RD, Zadorozny EV, Guo L, Tourkova IL and Blair HC (2009) Estrogen inhibits RANKL-stimulated osteoclastic differentiation of human monocytes through estrogen and RANKL-regulated interaction of estrogen receptor-alpha with BCAR1 and Traf6. *Exp Cell Res* **315:**1287-1301.

Romero JA, Zatz M and Axelrod J (1975) Beta-adrenergic stimulation of pineal N-acetyltransferase: adenosine 3':5'-cyclic monophosphate stimulates both RNA and protein synthesis. *Proc Natl Acad Sci U S A* **72:**2107-2111.

Roseboom PH NM, Zimonjic DB, Popescu NC, Rodriguez IR, Gastel JA, Klein DC (1998) Natural melatonin 'knockdown' in C57BL/6J mice: rare mechanism truncates serotonin N-acetyltransferase. *Brain Res Mol Brain Res.* **63:**189-197.

Rosen CJ and Bilezikian JP (2001) Clinical review 123: Anabolic therapy for osteoporosis. *J Clin Endocrinol Metab* **86:**957-964.

Ross AW, Barrett P, Mercer JG and Morgan PJ (1996) Melatonin suppresses the induction of AP-1 transcription factor components in the pars tuberalis of the pituitary. *Mol Cell Endocrinol* **123:**71-80.

Roth JA, Kim BG, Lin WL and Cho MI (1999) Melatonin promotes osteoblast differentiation and bone formation. *J Biol Chem* **274:**22041-22047.

Roy D, Angelini NL, Fujieda H, Brown GM and Belsham DD (2001) Cyclical regulation of GnRH gene expression in GT1-7 GnRH-secreting neurons by melatonin. *Endocrinology* **142:**4711-4720.

Rozen S and Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers., in: *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* (S K and S M eds), Humana Press.

Sack RL, Lewy AJ, Erb DL, Vollmer WM and Singer CM (1986) Human melatonin production decreases with age. *J Pineal Res* **3:**379-388.

Sadat-Ali M, al-Habdan I and al-Othman A (2000) Adolescent idiopathic scoliosis. Is low melatonin a cause? *Joint Bone Spine* **67:**62-64.

Saintier D, Khanine V, Uzan B, Ea HK, de Vernejoul MC and Cohen-Solal ME (2006) Estradiol inhibits adhesion and promotes apoptosis in murine osteoclasts in vitro. *J Steroid Biochem Mol Biol* **99:**165-173.

Sanchez-Rodriguez MA, Ruiz-Ramos M, Correa-Munoz E and Mendoza-Nunez VM (2007) Oxidative stress as a risk factor for osteoporosis in elderly Mexicans as characterized by antioxidant enzymes. *BMC Musculoskelet Disord* **8:**124.

Sandyk R, Anastasiadis PG, Anninos PA and Tsagas N (1992) Is postmenopausal osteoporosis related to pineal gland functions? *Int J Neurosci* **62:**215-225.

Satomura K, Tobiume S, Tokuyama R, Yamasaki Y, Kudoh K, Maeda E and Nagayama M (2007) Melatonin at pharmacological doses enhances human osteoblastic differentiation in vitro and promotes mouse cortical bone formation in vivo. *J Pineal Res* **42:**231-239.

Schomerus C and Korf HW (2005) Mechanisms regulating melatonin synthesis in the mammalian pineal organ. *Ann N Y Acad Sci* **1057:**372-383.

Schwartz MD, Wotus C, Liu T, Friesen WO, Borjigin J, Oda GA and de la Iglesia HO (2009) Dissociation of circadian and light inhibition of melatonin release through forced desynchronization in the rat. *Proc Natl Acad Sci U S A* **106:**17540-17545.

Sciaudone M, Gazzerro E, Priest L, Delany AM and Canalis E (2003) Notch 1 impairs osteoblastic cell differentiation. *Endocrinology* **144:**5631-5639.

Sethi S, Adams W, Pollock J and Witt-Enderby PA (2008) C-terminal domains within human MT1 and MT2 melatonin receptors are involved in internalization processes. *J Pineal Res* **45:**212-218.

Sharma R, Ottenhof T, Rzeczkowska PA and Niles LP (2008) Epigenetic targets for melatonin: induction of histone H3 hyperacetylation and gene expression in C17.2 neural stem cells. *J Pineal Res* **45:**277-284.

Shiga M, Kapila YL, Zhang Q, Hayami T and Kapila S (2003) Ascorbic acid induces collagenase-1 in human periodontal ligament cells but not in MC3T3-E1 osteoblast-like cells: potential association between collagenase expression and changes in alkaline phosphatase phenotype. *J Bone Miner Res* **18:**67-77.

Siddappa R, Mulder W, Steeghs I, van de Klundert C, Fernandes H, Liu J, Arends R, van Blitterswijk C and de Boer J (2009) cAMP/PKA signaling inhibits osteogenic differentiation and bone formation in rodent models. *Tissue Eng Part A* **15:**2135-2143.

Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP and Pacifici R (2001) Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-kappa B ligand (RANKL)-induced JNK activation. *J Biol Chem* **276:**8836-8840.

Strathdee G, Sim A and Brown R (2004) Control of gene expression by CpG island methylation in normal cells. *Biochem Soc Trans* **32:**913-915.

Suzuki A, Palmer G, Bonjour JP and Caverzasio J (1999) Regulation of alkaline phosphatase activity by p38 MAP kinase in response to activation of Gi protein-coupled receptors by epinephrine in osteoblast-like cells. *Endocrinology* **140:**3177-3182.

Suzuki N and Hattori A (2002) Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. *J Pineal Res* **33:**253-258.

Suzuki N, Somei M, Kitamura K, Reiter RJ and Hattori A (2008a) Novel bromomelatonin derivatives suppress osteoclastic activity and increase osteoblastic activity: implications for the treatment of bone diseases. *J Pineal Res* **44:**326-334.

Suzuki N, Somei M, Seki A, Reiter RJ and Hattori A (2008b) Novel bromomelatonin derivatives as potentially effective drugs to treat bone diseases. *J Pineal Res* **45:**229-234.

Tan DX, Manchester LC, Terron MP, Flores LJ, Tamura H and RJ R (2007) Melatonin as a naturally occurring co-substrate of quinone reductase-2, the putative MT3 melatonin membrane receptor: hypothesis and significance. *J Pineal Res* **43:**317-320.

Tan DX, Manchester LC, Reiter RJ, Qi WB, Zhang M, Weintraub ST, Cabrera J, Sainz RM and Mayo JC (1999) Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. *Biochim Biophys Acta* **1472:**206-214.

Tan DX, Manchester LC, Terron MP, Flores LJ, Tamura H and Reiter RJ (2007) Melatonin as a naturally occurring co-substrate of quinone reductase-2, the putative MT3 melatonin membrane receptor: hypothesis and significance. *J Pineal Res* **43:**317-320.

Tanaka K, Nagayama Y, Nishihara E, Namba H, Yamashita S and Niwa M (1998) Palmitoylation of human thyrotropin receptor: slower intracellular trafficking of the palmitoylation-defective mutant. *Endocrinology* **139:**803-806.

Tang SY (2008) [Effect of bone morphogenetic protein-2 on the expression of membrane Type 1-matrix metalloproteinase in human A549 lung carcinoma cells]. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* **33:**634-637.

Tengattini S, Reiter RJ, Tan DX, Terron MP, Rodella LF and Rezzani R (2008) Cardiovascular diseases: protective effects of melatonin. *J Pineal Res* **44:**16-25.

Tezuka K, Yasuda M, Watanabe N, Morimura N, Kuroda K, Miyatani S and Hozumi N (2002) Stimulation of osteoblastic cell differentiation by Notch. *J Bone Miner Res* **17:**231-239.

Thomas D and Kansara M (2006) Epigenetic modifications in osteogenic differentiation and transformation. *J Cell Biochem* **98:**757-769.

Titorencu I, Jinga VV, Constantinescu E, Gafencu AV, Ciohodaru C, Manolescu I, Zaharia C and Simionescu M (2007) Proliferation, differentiation and characterization of osteoblasts from human BM mesenchymal cells. *Cytotherapy* **9:**682-696.

Turgut M, Kaplan S, Turgut AT, Aslan H, Guvenc T, Cullu E and Erdogan S (2005) Morphological, stereological and radiological changes in pinealectomized chicken cervical vertebrae. *J Pineal Res* **39:**392-399.

Uebelhart D, Frey D, Frey-Rindova P, Goerres G and Michel BA (2003) [Therapy of osteoporosis: bisphosphonates, SERM's, teriparatide and strontium]. *Z Rheumatol* **62:**512-517.

Uslu S, Uysal A, Oktem G, Yurtseven M, Tanyalcin T and Basdemir G (2007) Constructive effect of exogenous melatonin against osteoporosis after ovariectomy in rats. *Anal Quant Cytol Histol* **29:**317-325.

van Straalen JP, Sanders E, Prummel MF and Sanders GT (1991) Bone-alkaline phosphatase as indicator of bone formation. *Clin Chim Acta* **201:**27-33.

von Gall C, Stehle JH and Weaver DR (2002) Mammalian melatonin receptors: molecular biology and signal transduction. *Cell Tissue Res* **309:**151-162.

Wada T, Nakashima T, Hiroshi N and Penninger JM (2006) RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* **12:**17-25.

Waldhauser F, Weiszenbacher G, Frisch H, Zeitlhuber U, Waldhauser M and Wurtman RJ (1984) Fall in nocturnal serum melatonin during prepuberty and pubescence. *Lancet* **1:**362-365.

Weaver DR (1998) The suprachiasmatic nucleus: a 25-year retrospective. *J Biol Rhythms* **13:**100-112.

Witt-Enderby PA, Bennett J, Jarzynka MJ, Firestine S and Melan MA (2003) Melatonin receptors and their regulation: biochemical and structural mechanisms. *Life Sci* **72:**2183-2198.

Witt-Enderby PA and Dubocovich ML (1996) Characterization and regulation of the human ML1A melatonin receptor stably expressed in Chinese hamster ovary cells. *Mol Pharmacol* **50:**166-174.

Witt-Enderby PA, MacKenzie RS, McKeon RM, Carroll EA, Bordt SL and Melan MA (2000) Melatonin induction of filamentous structures in non-neuronal cells that is dependent on expression of the human mt1 melatonin receptor. *Cell Motil Cytoskeleton* **46:**28-42.

Witt-Enderby PA, Radio NM, Doctor JS and Davis VL (2006) Therapeutic treatments potentially mediated by melatonin receptors: potential clinical uses in the prevention of osteoporosis, cancer and as an adjuvant therapy. *J Pineal Res* **41:**297-305.

Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD and Franceschi RT (2002) Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase

signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res* **17:**101-110.

Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G and Franceschi RT (2000) MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem* **275:**4453-4459.

Yang S, Madyastha P, Bingel S, Ries W and Key L (2001) A new superoxide-generating oxidase in murine osteoclasts. *J Biol Chem* **276:**5452-5458.

Yoshikubo H, Suzuki N, Takemura K, Hoso M, Yashima S, Iwamuro S, Takagi Y, Tabata MJ and Hattori A (2005) Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. *Life Sci* **76:**2699-2709.

Zaminy A, Ragerdi Kashani I, Barbarestani M, Hedayatpour A, Mahmoudi R and Farzaneh Nejad A (2008) Osteogenic differentiation of rat mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells: melatonin as a differentiation factor. *Iran Biomed J* **12:**133-141.

Zamurovic N, Cappellen D, Rohner D and Susa M (2004) Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. *J Biol Chem* **279:**37704-37715.

Zawilska JB, Skene DJ and Arendt J (2009) Physiology and pharmacology of melatonin in relation to biological rhythms. *Pharmacol Rep* **61:**383-410.

zur Nieden NI, Kempka G and Ahr HJ (2003) In vitro differentiation of embryonic stem cells into mineralized osteoblasts. *Differentiation* **71:**18-27.

.

12. APPENDIX

12.1 Sample Calculations for measuring ALP Activity in hAMSCs

	well 1 405 nM	well 2 405 nM	average	average- blank
blank	0.048	0.054	0.051	0
0	0.075	0.077	0.076	0.025
12.5	0.196	0.169	0.182	0.131
25	0.329	0.322	0.326	0.275
37.5	0.459	0.45	0.459	0.408
50	0.572	0.561	0.567	0.516
62.5	0.673	0.696	0.685	0.634
75	0.78	0.837	0.813	0.762

Table 2: Standard Curve Values Depicting the Rate of Hydrolysis of p-Nitrophenyl Phosphate Disodium Hexahydrate

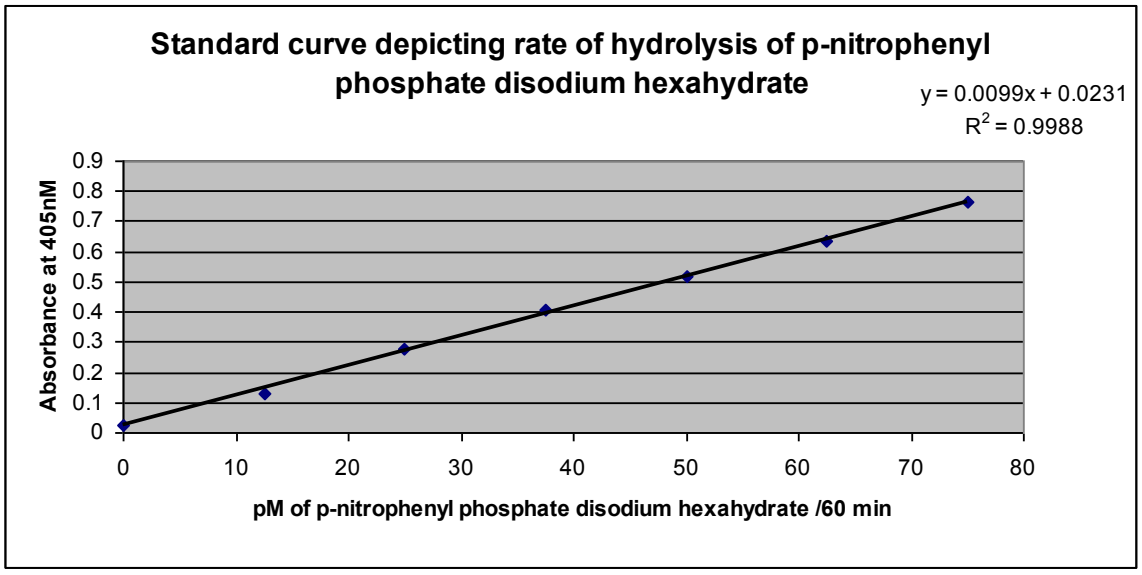


Figure 23: Standard Curve Depicting the Rate of Hydrolysis of p-Nitrophenyl Phosphate Disodium Hexahydrate

µg protein	well 1 405 nM	well 2 405 nM	well 3 405 nM	average	average-blank
blank	0.105	0.109	0.107	0.107	0.000
0	0.117	0.117	0.108	0.114	0.014
5	0.127	0.126	0.127	0.127	0.027
10	0.142	0.133	0.128	0.134	0.034
20	0.151	0.145	0.141	0.146	0.046
30	0.156	0.152	0.139	0.149	0.049
40	0.170	0.166	0.167	0.168	0.068
50	0.181	0.191	0.186	0.186	0.086
60	0.199	0.199	0.204	0.201	0.101
100	0.236	0.234	0.223	0.231	0.131
200	0.333	0.338	0.355	0.342	0.242

Table 3: Standard Curve Values Depicting Total Protein via BSA Assay

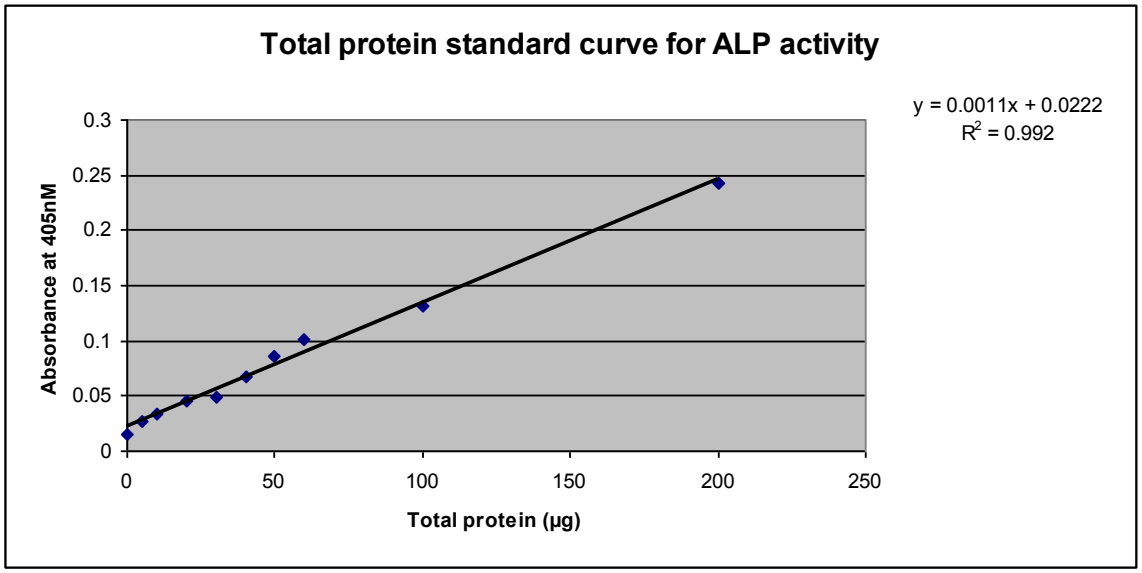


Figure 24: Standard Curve Depicting Total Protein

	Well 1 405 nM	Well 2 405 nM	Well 3 405 nM	Average	Average -blank	Rate of hydrolysis of p-nitrophenyl phosphate disodium hexahydrate calculated from Fig. 23	Total protein calculated from Fig. 24	Rate of hydrolysis/ Total protein	% control (ALP activity)
control	0.40	0.42	0.54	0.45	0.40	38.76	37.15	1.04	100.0
21d	2.25	2.19	2.19	2.22	2.17	217.00	49.96	4.34	416.20

Table 5: Sample ALP Activity Calculation of Control vs. 21 d Melatonin Exposure

12.2 Sample Calculations for Quantitative Analysis of Osteogenesis

µM	well 1	well 2	well 3	average	average - blank
blank	0.045	0.045	0.046	0.045	0.000
2000.00	1.646	1.691	1.663	1.667	1.622
1000.00	0.894	0.889	0.887	0.890	0.845
500.00	0.489	0.485	0.493	0.489	0.444
250.00	0.278	0.284	0.287	0.283	0.238
125.00	0.161	0.176	0.171	0.169	0.124
62.50	0.107	0.113	0.108	0.109	0.064
31.30	0.080	0.078	0.078	0.079	0.034
15.00	0.076	0.078	0.077	0.077	0.032
7.50	0.061	0.061	0.061	0.061	0.016
3.75	0.053	0.053	0.054	0.053	0.008
1.88	0.049	0.051	0.048	0.050	0.005
0.94	0.047	0.049	0.046	0.047	0.002

Table 6: Standard Curve Values Depicting Colorimetric Analysis of Known Concentrations of Alizarin Red Solutions

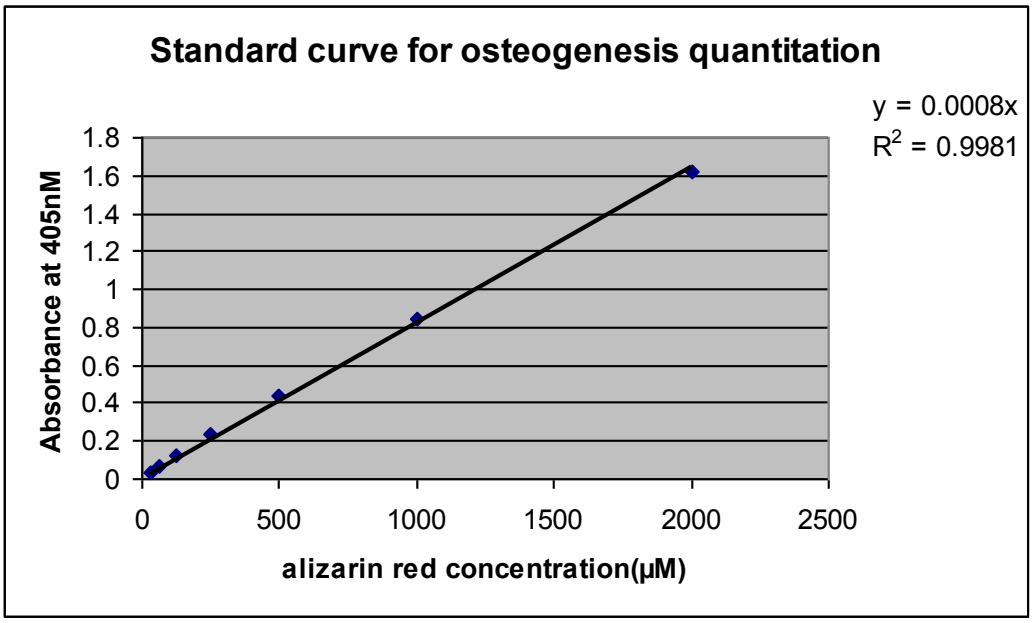


Figure 25: Standard Curve Depicting Colorimetric Analysis of Known Concentrations of Alizarin Red Solutions

	well 1 405 nM	well 2 405 nM	well 3 405 nM	average	average - blank	quantitation of samples using Fig. 26	% control
control	0.143	0.141	0.121	0.135	0.090	112.500	100.000
21d	0.928	0.887	0.923	0.913	0.868	1084.417	963.926

Table 7: Sample Calculation for Quantitative Analysis of Osteogenesis of Control vs. 21 d Melatonin Exposure

12.3 Sample Calculations for Real- Time RT- PCR

Real-time PCR results were analyzed using the $\Delta\Delta C_t$ method as described by Livak & Schmittgen (2001). The step by step representation of this method is given below.

1. *Calculation of C_t value:* C_t values represent the cycle number. The C_t values were calculated from the PCR graph by extrapolating the threshold value (set at 100) to X axis (see representative graph in Fig. 27).

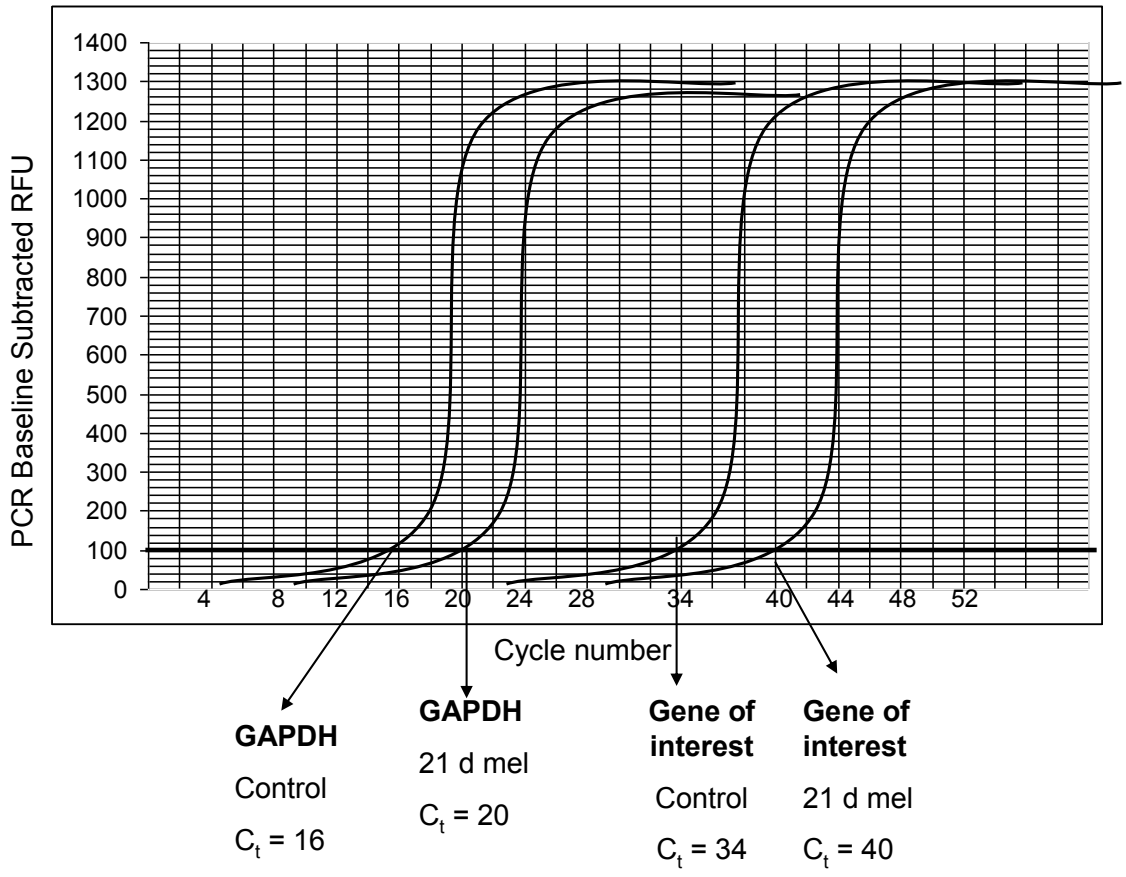


Figure 26: Sample Real-time PCR Graph Depicting C_t values for GAPDH and Gene of Interest

2. *Determination of threshold value:* The threshold value was set at a value above the background fluorescence but within the linear phase. Fig. 28 illustrates the logarithmic graph view of the PCR data. The fluorescence threshold value was set at 100 RFU.

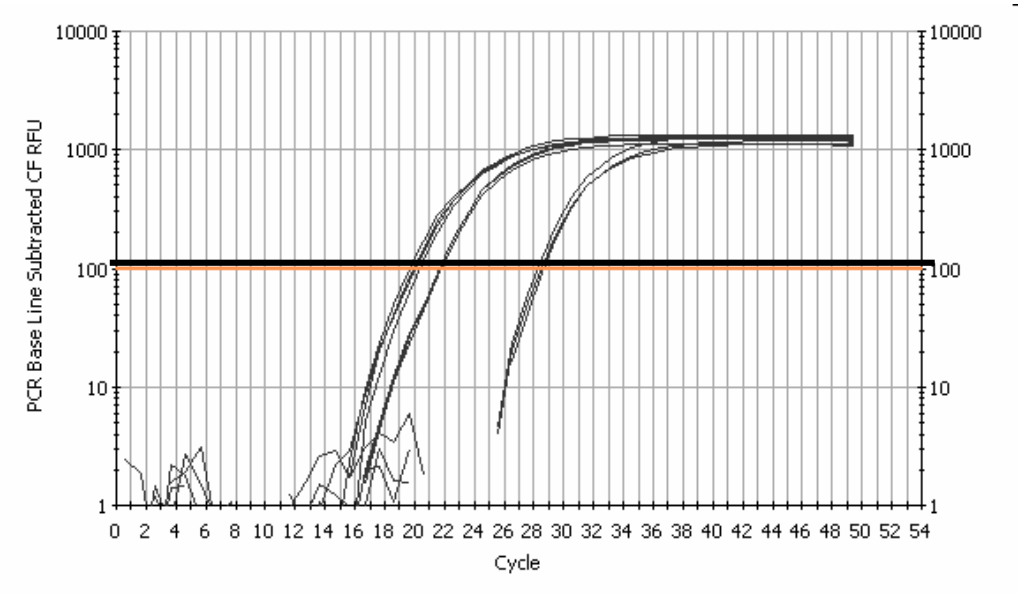


Figure 27: Logarithmic Graph View of Example Real-Time PCR data
The fluorescence threshold value was set at 100 RFU.

3. *Calculation of fold change in gene expression over control:* The ΔC_t values were calculated by subtracting the C_t value of the housekeeping gene (*GAPDH*) from the C_t value of the gene of interest. A lower C_t value indicates higher gene expression. The ΔC_t values in each group were averaged and used to calculate the relative fold change in expression ($2^{-\Delta\Delta Ct}$).

	Gene of interest C_t	*GAPDH* C_t	ΔC_t (gene of interest-GAPDH)	$(\Delta \Delta C_t)$ (treatment – control)	$2^{-(\Delta \Delta Ct)}$ Fold change
control	34	16	18	0	1
21d mel	40	20	20	2	0.25

Table 8: Calculation of Fold Change in Gene Expression with 21 d Melatonin Exposure over Control

4. *Melt curve analysis*: A melt curve analysis was performed after the real-time PCR runs. A melt curve detects the melting temperature of the product in the reaction well. Each PCR product has a specific melting temperature and a single melt peak indicates the presence of a single product.

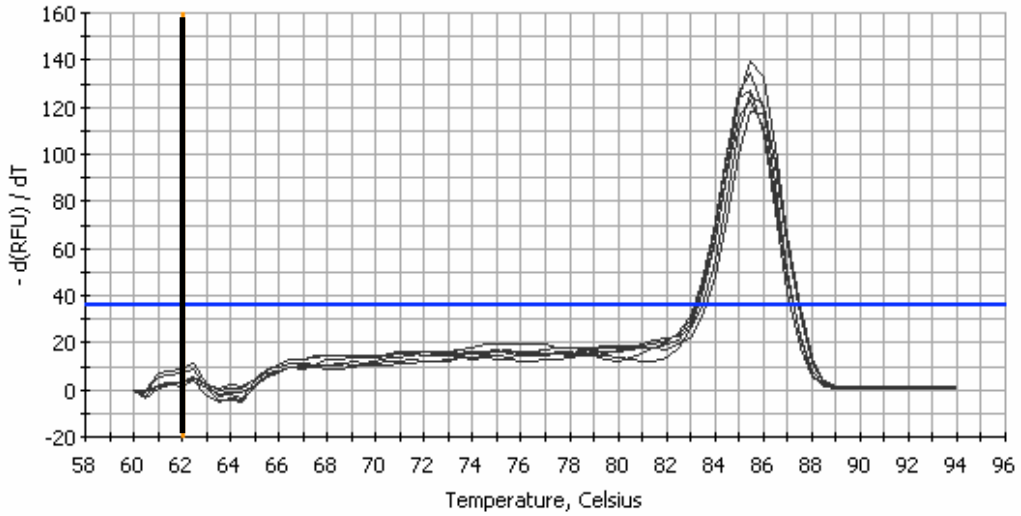


Figure 28: Sample Real-Time PCR Melt Curve