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A Mechanistic Evaluation of how Melatonin Enhances Alkaline Phosphatase Activity in Human Adult Mesenchymal Stem Cells

Nicholas Radio

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**A MECHANISTIC EVALUATION OF HOW MELATONIN ENHANCES
ALKALINE PHOSPHATASE ACTIVITY IN
HUMAN ADULT MESENCHYMAL STEM CELLS**

A Dissertation

Presented to the Graduate School of Pharmaceutical Sciences

Of

Duquesne University

In Partial Fulfillment of the
Requirements for the Degree

Of

Doctor of Philosophy
Pharmacology/Toxicology

By

Nicholas Michael Radio

March 3rd, 2006

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Dissertation: A Mechanistic Evaluation of how Melatonin Enhances Alkaline Phosphatase Activity in Human Adult Mesenchymal Stem Cells

Degree: Doctor of Philosophy

Date: March 3rd, 2006

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I. Introduction

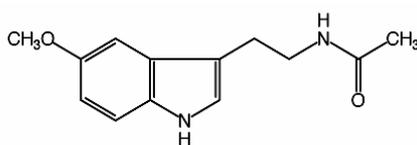
A. Statement of Problem

The structural integrity of bone is dependent upon a balance between the activity of bone-resorbing osteoclasts and bone-forming osteoblasts [33, 34]. Through this remodeling process, bone is constantly degraded and replaced with new bone. As humans age, osteoblast activity begins to diminish due to reduced reproductive and biosynthetic potential [35]. The resulting imbalance in osteoblast to osteoclast activity results in local or systemic bone loss called osteoporosis [36], which affects 28 million Americans [37]. As one of the prominent health problems associated with aging, osteoporosis is most prevalent in post-menopausal women as a result of decreased estrogen levels, but is also common in aging men. A number of therapeutic approaches are available to relieve the symptoms of osteoporosis, however, most target osteoclasts to prevent further bone loss (i.e., raloxifene, EVISTA; alendronate FOSAMAX; calcitonin MIACALCIN) [38]. Another approach is to stimulate the formation of bone by targeting the proliferation, differentiation, and activity of osteoblasts [39] using teriparatide PTH 1-34 (FORTEO). The hormone melatonin has recently been shown to enhance osteoblast differentiation in animal cell models [43]. The goals of this project are therefore to determine whether melatonin enhances osteoblast differentiation in a human cell model, and if so, determine the signaling pathways melatonin utilizes to cause these enhancing effects.

B. Literature Survey

1. Synthesis of Melatonin

The peptide melatonin is the principle hormone produced from the pineal gland through a four step enzymatic transformation originating with the aromatic amino acid tryptophan. Tryptophan is transported into the brain through an active uptake process specific to large, neutral amino acids [1, 2]. Once inside the brain, tryptophan is hydroxylated by tryptophan hydroxylase, to form 5-hydroxytryptophan and rapidly decarboxylated by aromatic amino acid decarboxylase to form serotonin. The pineal gland is located along the roof of the thalamus, separated from the brain by the blood brain barrier. Despite its separation from the brain, the pineal gland contains all of the enzymes necessary to produce serotonin and has 50 times more serotonin content than the entire brain [1]. Inside the pineal gland, melatonin is synthesized from serotonin by *N*-acetyltransferase activity to form *N*-acetyl serotonin, which is acted on by 5-hydroxy indole *O*-methyltransferase to form melatonin [1, 2].



Melatonin

The production of *N*-acetyltransferase, the rate-limiting enzyme necessary to convert serotonin to melatonin, is modulated by both lighting conditions and circadian oscillation patterns [2]. Production of *N*-acetyltransferase is 15 to 30-fold higher during periods of darkness [3] and light exposure as brief as 1 min reduces *N*-acetyltransferase activity by over 50% [4]. The pineal gland's

sensitivity to lighting conditions is detected by the eye and occurs through central innervation [5]. Light sensitivity is maintained despite interruption of the sympathetic innervation via removal of the superior cervical ganglia [5]. Independent of lighting conditions, *N*-acetyltransferase production is also influenced via a cyclic daily rhythm [6]. Like *N*-acetyltransferase production, the clock genes *Per1* and *Cry2* in the rat pineal gland are expressed in a circadian fashion and regulated by the circadian-driven changes in norepinephrine [7]. Within the chicken pineal gland, the enzymes necessary for melatonin synthesis, namely tryptophan hydroxylase, arylalkylamine-*N*-acetyltransferase, and hydroxyindole-*O*-methyl-transferase mRNA are expressed in a circadian fashion that persists during periods of constant darkness [8]. Furthermore, in blind mammals, *N*-acetyltransferase activity is continuously produced on a cyclic pattern, but is independent of lighting conditions [1].

Both the light-dark cycle and circadian inductions in *N*-acetyltransferase production are mediated through sympathetic stimulation [3, 6]. During periods of darkness, norepinephrine containing sympathetic nerves are stimulated, inducing the release of norepinephrine from nerve endings within the pineal gland, whereupon it binds to β_1 -adrenergic receptors coupled to G_s proteins, or α_1 -adrenergic receptors coupled to G_q proteins [9, 10]. Upon β_1 receptor activation, G_s proteins activate adenylyl cyclase located within the transmembrane. Once activated, adenylyl cyclase stimulates the conversion of adenosine triphosphate to cyclic AMP production and subsequent allosteric PKA activation. Additionally, upon α_1 receptor activation, G_q activates PKC activity,

causing an increased release of Ca^{2+} from the endoplasmic reticulum followed by a rapid influx of Ca^{2+} into the pinealocyte [10]. The induction of activated PKA and increased Ca^{2+} concentration leads to the activation of CREB transcription factors, which induces the transcriptional production of *N*-acetyltransferase enzyme. While both β_1 and α_1 adrenergic receptor activation stimulates *N*-acetyltransferase production, β_1 receptor can independently stimulate *N*-acetyltransferase production [9], while α_1 receptor activation preferentially stimulates serotonin release from the pineal gland [11].

2. Metabolism of Melatonin

Melatonin metabolism occurs primarily through conjugation reactions (~70%) and secondarily through oxidation (~15%) or alternatively, it is excreted unchanged (15%) [12]. Prior to conjugation, melatonin must be hydroxylated to form 6-hydroxy-melatonin or demethylated to form *N*-acetyl-serotonin, which are then conjugated with sulphur or glucuronide to form the principle melatonin metabolites, 6-hydroxy-melatonin sulphate or *N*-acetyl-serotonin, respectively [12]. Hydroxylation and demethylation of melatonin is mediated through the high-affinity cytochrome p450 enzyme isoform CYP1A2 within the liver [13]. Alternatively, oxidative metabolism of melatonin metabolism may proceed through two different enzymes, with the route dependent upon the presence or absence of the peroxide cofactor [14]. When peroxide is present, melatonin is oxidized via indoleamine 2, 3-dioxygenase, which is also capable of tryptophan and serotonin oxidation. When peroxide is absent, indoleamine 2, 3-dioxygenase is incapable of oxidizing melatonin, leaving myeloperoxidase as the

sole enzyme capable of oxidizing melatonin by cleaving the indole moiety. Potential metabolites formed during melatonin oxidation are *N*¹-acetyl-*N*²-formyl-5-methoxykynuramine (AFMK) and *N*¹-acetyl-5-methoxykynuramine (AMK). Both metabolites have demonstrated antioxidant and free radical scavenging abilities that may further amplify melatonin's antioxidant actions [15].

3. Subtypes of the Melatonin Receptor

Melatonin may act through a variety of mediators, including melatonin G-protein coupled receptors, quinone reductase coupled melatonin receptors, or alternatively, melatonin can act independently of a receptor due to its lipophilic nature [16] and potent antioxidant properties [15, 17]. Two distinct subtypes of melatonin seven transmembrane G protein-coupled receptors, designated MT₁ and MT₂ have been cloned and characterized. Both G protein-coupled receptors are coupled to G_i and G_q proteins [18, 31, 32], that upon activation, induces an inhibition of adenylyl cyclase to reduce cyclic AMP levels (0.1 pM – 1 nM) or phospholipase C activation, which hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP₂) to form inositol 1, 4, 5-trisphosphate and diacylglycerol, respectively (1 nM – 1 μM) [19, 32]. The MT₁ melatonin receptor is widely dispersed throughout the body, including the suprachiasmatic nucleus (SCN), pars tuberalis, retina and cardiac blood vessels, where physiologically, melatonin inhibits neuronal firing [20], inhibits prolactin secretion on a circadian basis [21], modulates retinal physiology [22], and dilates blood vessels, respectively [30, 32]. Alternatively, the MT₂ melatonin receptor, in addition to coupling with G_i or G_q proteins, is also able to inhibit the soluble guanylyl cyclase pathway [19, 32].

The MT_2 melatonin receptor is localized within the SCN, cardiac vessels, kidney, ovary, and retina where it modulates circadian rhythms, constricts blood vessels, differentiates the nephron [23], induces folliculogenesis [24], and inhibits dopamine release [25], respectively. A second type of melatonin receptor, designated MT_3 , has more recently been identified in hamster kidney or ovary cells that shares 95% homology to the human quinone reductase 2 enzyme [26]. More recent findings indicate the MT_3 binding site is diversely present in a wide array of mammals and tissue, with highest receptor amounts and quinone reductase enzymatic activity uniformly detected in the liver and kidney [27]. Functionally, the quinone reductase 2 enzyme is responsible for the reduction of quinones to stable intermediates that can be later conjugated and excreted, thus preventing the production of highly reactive oxygen species [28]. Additionally, quinone reductase 2 has demonstrated antioxidant abilities that may partially explain melatonin's observed antioxidant effects [17]. Physiologically, MT_3 melatonin receptor activation has been shown to decrease intraocular pressure and inhibit adhesion of leukocytes [29, 30].

4. Melatonin and Osteoporosis

Mounting evidence associates the age-related decreases in plasma melatonin to increased susceptibility to osteoporosis [40]. Melatonin plasma levels are significantly lower in humans past the age of 50 compared to pre-pubescent individuals [41-43] and these age-related reductions may lead to the increased occurrence of osteoporosis throughout adulthood [40, 43-45]. Recently Turgut and colleagues [46] showed that pinealectomized chickens had

significantly lower bone mineral density at the cervical vertebral body compared with nonpinealectomized control animals. Pinealectomized chickens also had a plateau-shaped density histogram between the fourth and seventh cervical vertebra indicating an inactive osteoblast population compared with nonpinealectomized animals with higher density bone tissues. Histological changes were also noted in the pinealectomized chickens, as the total number of osteocytes were significantly lower than control, suggesting melatonin enhances osteocyte proliferation in the cervical vertebra. Several animal models have reported that melatonin has a preventive effect of idiopathic scoliosis [46-50]. This demonstrated correlation appears independent of posture and gravity, because pinealectomized Atlantic salmon have abnormal spinal curves, reduced mechanical properties, and reduced vertebral mineral content [47]. The effect of melatonin on cellular differentiation has stimulated interest in its role in osteoporosis.

Melatonin accumulation and/or synthesis within the bone marrow has been widely documented *in vivo*, where it may act to enhance bone formation [51-54]. Elevated melatonin concentrations occur in the bone marrow of rats, with night-time bone marrow concentrations approximately twice as high as the peripheral blood [51]. Exogenously administered melatonin collects within the bone marrow against a plasma concentration gradient, suggesting the presence of melatonin binding proteins. Although lower than pineal intact rats, elevated bone marrow melatonin concentrations persist in long-term pinealectomized rats. These data indicate that a portion of melatonin present in the bone marrow may

come from an extrapineal source. Additionally, enzymes necessary to convert serotonin to melatonin, namely *N*-acetyltransferase (NAT), but not hydroxyindole-O-methoxyltransferase (HIOMT) activity are present in the bone marrow cells. Various potential mechanisms exist regarding how melatonin enhances bone formation.

Melatonin is implicated in bone development through both the suppression of bone-resorbing osteoclast cells [45, 54], possibly via its free radical scavenging properties [55-59], and through the enhancement of bone producing osteoblast differentiation [43, 44]. Melatonin causes a dose-dependent reduction in both osteoclastogenesis and osteoclast activity by reducing the number of resorption pits and average pit area/pit in mouse bone marrow cells possibly through the down-regulation of receptor activator of NF- κ B ligand (RANKL) and hence NF- κ B activity [45]. Furthermore, in goldfish scales, melatonin significantly suppresses osteoclast activity, observed via a reduction in tartrate-resistant acid phosphatase (TRACP) activity following six hour incubation with 10 nM, 1 μ M, and 10 μ M melatonin [54].

Besides having effects on osteoclasts, several studies show that melatonin enhances bone formation through the stimulation of bone-producing osteoblast cells [43, 44]. Melatonin's ability to directly promote osteoblast maturation was first demonstrated in pre-osteoblast and rat osteoblast-like osteosarcoma cells, [43] where low (10 nM) concentrations of melatonin increase the mRNA levels of several genes expressed in osteoblasts including alkaline phosphatase, bone sialoprotein (BSP), osteopontin, and osteocalcin.

Furthermore, these osteoblast effects are mediated through melatonin transmembrane receptors, as exposure to pertussis toxin or the melatonin receptor antagonist luzindole significantly reduces bone sialoprotein and alkaline phosphatase expression [43]. Similar results are reported in clinically relevant human bone cells, in which micromolar concentrations of melatonin significantly increase procollagen type I c-peptide production in a concentration-dependent manner [44].

Even though a majority of the reports show positive effects on bone formation, in some studies, inverse correlations between melatonin and bone formation are observed. For example, melatonin suppresses the activity of osteoblast cells, possibly via the down-regulation of estrogen receptor and insulin-like growth factor (IGF)-1 mRNA expression. Ostrowska and colleagues report similar findings, as alkaline phosphatase (ALP), carboxyterminal propeptide of type I procollagen (PICP), and cross-linked carboxyterminal telopeptide of type I collagen (ICTP) were negatively correlated with endogenous melatonin concentrations in male Wistar rats [58]. Additionally, pinealectomy in male Wistar rats increases the levels of bone metabolism biomarkers, including alkaline phosphatase activity, carboxyterminal propeptide of type I procollagen, and cross-linked carboxyterminal telopeptide of type I collagen [59]. Similarly, melatonin and estradiol co-exposure lowers urinary deoxypyridinoline, a marker of bone resorption in ovariectomized rats [60].

5. Signal Transduction Mechanisms Mediating Osteoblast Differentiation

The underlying signal transduction mechanism(s) responsible for

melatonin's effects on osteoblast differentiation have yet to be elucidated, but evidence suggests that the mitogen activated protein kinase (MAP-K) signal transduction pathway may be involved [61-67]. Supporting this, Rawadi and co-workers [65] find that osteoblast differentiation is dependent upon MEK (1/2) activation, as selective MEK inhibitors (PD98059, U0126) block osteoblast differentiation in either a murine mesenchymal stem cell line or a pluripotent murine myoblast cell line induced by a phosphodiesterase inhibitor. In human adult mesenchymal stem cells (hAMSCs), UO126 co-incubated with EGF ligand, completely eliminates phosphorylation of ERK1 and ERK2, with subsequent inhibition of the hAMSC converting to the osteoblast [67]. Similarly, Suzuki and colleagues [63] find that the MAP-Ks, ERK and p38, and potentially JNK, regulate different stages of osteoblast cell proliferation and differentiation.

Phosphorylation of the MAP-K signaling cascade may also be mediated by the epidermal growth factor receptor (EGFR). In this regard, transactivation of the EGFR by G-protein coupled receptors may occur through a matrix metalloproteinase-mediated proteolytic release of EGF. In human adult mesenchymal stem cells, EGFR induces the phosphorylation of ERK 1/2 and modulates osteoblast differentiation [67]. Probing further into the signaling mechanisms that underlie GPCR-mediated activation of ERK, it is found that G-proteins and matrix metalloproteinases may be involved. For example, using PC12 cells, Piiper and colleagues [68] demonstrate that EGFRs, activated by increases in cAMP, induce ERK 1/2 phosphorylation that is reversed in the presence of the EGFR inhibitor, PD165393. In addition, using renal mesangial

cells, the GPCRs 5-hydroxytryptamine_{2A}, lysophosphatidic acid, angiotensin AT₁ and bradykinin B₂, induce the phosphorylation of the EGFR and ERK through a PKC-dependent pathway [69]. Also, transactivation of the EGFR by G proteins is mediated by the G_{βγ} subunit in recombinant cells [70], or matrix metalloproteinases in transgenic murine osteoblasts [71]. Subsequent to G_i protein activation via lysophosphatidic acid exposure, it is shown that the G_{βγ} subunit induces the phosphorylation of Shc adapter proteins; proteins that bind to tyrosine phosphoproteins like EGFRs. Phosphorylation of the EGFR and Shc adapter protein binding is reversible following cellular expression of dominant negative kinase-inactive mutants of c-Src. In a different study, G_i-protein coupled lysophosphatidic acid receptors induce ERK activation, and this increase in ERK activity coincided with EGFR phosphorylation [71]. EGFR phosphorylation is sensitive to matrix metalloproteinase inhibition, indicating EGFR transactivation occurs via proteolytic EGF ligand liberation [71]. Additionally, proteolysis of heparin-bound EGF ligand is shown to induce the autophosphorylation of EGFR and subsequent ERK activation. Taken together, these studies suggest melatonin-mediated changes in MEK 1/2 activity may occur through matrix metalloproteinase-mediated transactivation of the epidermal growth factor receptor.

A multifunctional group of proteins may facilitate melatonin mediated activation of epidermal growth factors and/or the MAP-K signaling cascade. β-arrestin-1 and -2 are ubiquitously distributed proteins classically involved with GPCR desensitization and clathrin-dependent internalization following prolonged

agonist exposure [72, 73]. As a means of stimulus-driven homologous desensitization, activated GPCRs are readily phosphorylated on serine or threonine residues by G protein-coupled receptor kinases (GRK), inducing their translocation to the plasma membrane and binding of β -arrestin-1 and β -arrestin-2 proteins to the carboxy terminal region of the GPCR [74]. Once bound, β -arrestin induces the uncoupling of the GPCR from the G-protein and targets the receptor for clathrin-dependent receptor internalization and possible degradation [69]. Besides the protein's receptor regulatory actions, β -arrestin has more recently been shown to act as a scaffolding protein; connecting downstream proteins to the receptor to facilitate signaling [75-79] and causing the activation of the MAP-K cascade [74, 79]. Through an intricate set of experiments, Luttrell and colleagues [76] found that angiotensin exposure induces the formation of multi-protein complexes, which include the angiotensin II type 1a (AT1aR) GPCR, β -arrestin-2, and the complete set of MAP-K proteins, cRaf-1, MEK1, and ERK2.

The functional consequences of GPCR desensitization and signaling ability is highlighted in GRK mutant cells. Osteoblastic cells expressing dominant negative GRK mutants reduce cellular proliferation and impair tyrosine kinase-mediated activation of the MAP-K signaling cascade [80]. Thus, impeding receptor phosphorylation and subsequent β -arrestin binding is detrimental to cellular function.

Interestingly, β -arrestin scaffolding proteins have also been implicated in both the activation and translocation patterns of MAP-K into both the cytosol [76]

and nuclear compartments [74]. The translocation patterns of ERK (i.e. cytosol vs. nucleus) may dictate the response of the cell to a stimulus. Because the MAP-K pathway is intimately involved in proliferative and differentiation responses, then the distribution of ERK to various cellular compartments may determine which of these responses will be affected. Unique ERK translocation patterns within a cell in response to different stimuli are demonstrated in several studies. Angiotensin exposure induces the redistribution of activated ERK2 into endosomal vesicles within the cytosol, while in COS-7 cells transfected with β_1 or β_2 adrenergic receptors, β -arrestin binding induces activated ERK translocation into the nucleus [74]. The β -arrestin-mediated variance within translocation patterns between the cytosol and nucleus may depend on the stability of the GPCR- β -arrestin interaction [79]. Stable GPCR- β -arrestin complexes enhance the activation of ERK within the cytosol, whereas transient receptor- β -arrestin complexes result in a decrease in ERK-P within the nucleus. The reduction in ERK-P within the nucleus correlates with a decrease in transcriptional and mitogenic responses, indicating that the stability of the GPCR- β -arrestin complex influences both ERK translocation and ERK-mediated responses.

β -Arrestin ubiquitination appears to regulate the receptor internalization and scaffolding properties of the protein. Following β_2 adrenergic receptor activation, Mdm2 and E3 ubiquitin ligase binds to and ubiquitinates β -arrestins. Inhibition of the Mdm2-mediated β -arrestin ubiquitination prevents both receptor internalization and enhances translocation of activated ERK into the cytosol [81-83]. The melatonin GPCR may similarly utilize β -arrestin to facilitate homologous

receptor internalization and/or scaffolding with the MAP-K signaling cascade.

6. Human Adult Mesenchymal Stem Cell Model

Human adult mesenchymal stem cells (hAMSCs) are bone marrow-derived multipotent precursors for a variety of mesoderm cell lineages [84]. Current cell culture techniques, through a utilization of specific growth factors, are known to induce the differentiation of the hAMSCs into chondrocyte, adipocyte, or osteoblast terminal cell lines [84-87]. hAMSCs have been shown to differentiate into an osteoblast lineage when incubated in a medium containing dexamethasone, ascorbic acid, and β -glycerophosphate [85]. Under these conditions, ALP enzymatic activity significantly increases following 8 – 12 day incubation, with subsequent mineral deposition occurring by 12 – 16 d [85]. The ability of hAMSCs to differentiate into an osteoblast when incubated with an osteogenic inducing medium is maintained throughout passages 1-10 [86]. Because of their ability to differentiate into terminal mesodermal cells, hAMSCs are currently being investigated for their clinical potential in tissue regeneration [88-91]. Additionally, the hAMSC model being investigated is clinically appropriate, considering the human, non-cancerous origin that has been used in other models [43].

The goals of this study were to determine if melatonin, acting through transmembrane melatonin receptors, enhances human adult mesenchymal stem cell differentiation into osteoblasts, and, if so, determine the intracellular signal transduction pathways that mediate this process. Based on previous findings, we hypothesized that melatonin, acting through melatonin GPCRs, is able to

enhance the differentiation of hAMSC into osteoblasts mediated through the transactivation of EGFRs and subsequent stimulation of the MAP-K signaling cascade.

II. MATERIALS AND METHODS

A. Materials and Equipment

1. Facilities

Laboratories – Mellon Hall of Science, Rooms 245, 262, 406, 414 and 454

Office – Mellon Hall of Science, Room 420

2. Cell lines

Human Adult Mesenchymal Stem Cells (HAMSCs)
Cambrex Bio Science Walkersville, Inc., Walkersville, MD

3. Chemicals and Drugs

Anti- β -Arrestin-1 (Goat), Lot No. D0903
Santa Cruz Biotechnology, Inc., Santa Cruz, CA

Anti- β -Arrestin-2 (Mouse), Lot No. E3105
Santa Cruz Biotechnology, Inc., Santa Cruz, CA

Anti-Epidermal Growth Factor Receptor-Phos (Mouse),
Lot No. B58671
Lab Vision Co., Fremont, CA

Anti-G_{ai-2} (Rabbit), Lot No. J139
Santa Cruz Biotechnology, Inc., Santa Cruz, CA

Anti-MT₁ Receptor (Human)
Dr. Ralf Jockers, Université Paris VII

Anti-MT₂ Receptor (Human)
Dr. Ralf Jockers, Université Paris VII

Anti-Mitogen Activated Protein Kinase (Rabbit), Lot No. 03J4513
Sigma Chemical Co., St. Louis, MO

Anti-Mitogen Activated Protein Kinase-Phos (Rabbit), Lot No. 16
Cell Signaling, Beverly, MA

Anti-Mitogen Activated Protein Kinase Kinase (Rabbit),
Lot No. 78K1039
Sigma Chemical Co., St. Louis, MO

Anti-Mitogen Activated Protein Kinase Kinase-Phos (Rabbit),
Lot No. 15
Cell Signaling, Beverly, MA

β -Glycerophosphate, Lot No. 50F-0269
Sigma Chemical Co., St. Louis, MO

Bromophenol Blue, Lot No. DB102341
American Bioanalytical, Natick, MA

Complete Mini protease inhibitor cocktail tablets
Roche Diagnostics Corp., Indianapolis, IN

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide;
thiazolyl blue
Sigma Chemical Co., St. Louis, MO

Digitonin
Sigma Chemical Co., St. Louis, MO

Donkey Anti-goat IgG, Horseradish Peroxidase
Santa Cruz Biotechnology, Inc., Santa Cruz, CA

DTT, Lot No. 037538
Fisher Scientific, Pittsburgh, PA

Dulbecco's Phosphate Buffered Solution, Ca/Mg-free
Invitrogen, Grand Island, NY

ECL Anti-mouse IgG, Horseradish Peroxidase linked whole antibody
Amersham Biosciences, Piscataway, NJ

ECL Anti-rabbit IgG, Horseradish Peroxidase linked whole antibody
Amersham Biosciences, Piscataway, NJ

EDTA, Lot No. 113HO862
Sigma Chemical Co., St. Louis, MO

EGTA, Lot No. 30K5420
Sigma Chemical Co., St. Louis, MO

Ethanol (95%)
Prepared from stock, Duquesne University, Pittsburgh, PA

Forskolin, Lot No. 044K7042

Sigma Chemical Co., St. Louis, MO

Glycerol (99.9%)
Sigma Chemical Co., St. Louis, MO

Glycine, Lot No. 70K0069
Sigma Chemical Co., St. Louis, MO

GM 6001, Lot No. B65127
Calbiochem, La Jolla, CA

5'-Guanylylimidodiphosphate, Lot No. 064K0163
Sigma Chemical Co., St. Louis, MO

HEPES, Lot No. 951465
Fisher Scientific, Pittsburgh, PA

Human Mesenchymal Stem Cell Growth Medium
Cambrex Bio Science Walkersville, Inc., Walkersville, MD

Human Mesenchymal Stem Cell Osteogenesis Induction Medium
Cambrex Bio Science Walkersville, Inc., Walkersville, MD

Hydrochloric Acid, Lot No. 983601
Fisher Scientific, Pittsburgh, PA

Isopropranol (99.5%)
Fisher Scientific, Pittsburgh, PA

2-[¹²⁵I]-iodomelatonin (specific activity 2200 Ci/mmol) – Lot No. EG31760
NEN Dupont, Boston, MA

KT5720, Lot No. B26653
Sigma Chemical Co, St. Louis, MO

Luzindole, Lot No. 1/13867
Tocris, Ballwin, MO

Magnesium Chloride, Lot No. 27H0828
Sigma Chemical Co., St. Louis, MO

Melatonin, Lot No. 128H0473
Sigma Chemical Co., St. Louis, MO

Methanol (99.9%), Lot No. 60K3751
Sigma Chemical Co., St. Louis, MO

Monodansyl Cadaverine, Lot No. 50K3250
Sigma Chemical Co., St. Louis, MO

Nonfat Dry Milk
Topco Associates, Inc., Skokie, IL

Nonidet P-40
Fisher Scientific, Pittsburgh, PA

PD168393, Lot No. B27116
Calbiochem, La Jolla, CA

PD98059, Lot No. B24069
Calbiochem, La Jolla, CA

Pertussis toxin, Lot No. 129H1330
Sigma Chemical Co., St. Louis, MO

PhosphoDetect Anti-Epidermal Growth Factor mAb (Mouse)
Calbiochem, La Jolla, CA

P-Nitrophenol Phosphate Stock, Lot No. 101K6115
Sigma Chemical Co., St. Louis, MO

Polyethylenimine, Lot No. 78H0692
Sigma Chemical Co., St. Louis, MO

Potassium Chloride, Lot No. 89F-0869
Sigma Chemical Co., St. Louis, MO

Potassium Phosphate Monobasic, Lot No. 113H0292
Sigma Chemical Co., St. Louis, MO

4P-PDOT, Lot No. 1/24467
Tocris, Ballwin, MO

Protein A Agarose Beads, Lot No. 045K1223
Sigma Chemical Co., St. Louis, MO

Rolipram, Lot No. 055K4602
Sigma Chemical Co., St. Louis, MO

Sodium Chloride, Lot No. 041752
Fisher Scientific, Pittsburgh, PA

Sodium Dodecyl Sulfate, Lot No. 064K0163
Sigma Chemical Co., St. Louis, MO

Sodium Hydroxide, Lot No. 109H0187
Sigma Chemical Co., St. Louis, MO

Sodium Orthovanadate
Sigma Chemical Co., St. Louis, MO

Trichloroacetic Acid
Sigma Chemical Co., St. Louis, MO

Triton X-100
Sigma Chemical Co., St. Louis, MO

Trizma Base, Lot No. 064K5452
Sigma Chemical Co., St. Louis, MO

Trizma Hydrochloride, Lot No. 49H5402
Sigma Chemical Co., St. Louis, MO

4. Materials

Bicinchoninic acid Protein Assay Kit
Pierce, Rockford, IL

Biomax Light Film
Eastman Kodak Co., Rochester, NY

BioRad Protein Assay Kit, Lot No.
BioRad, Hercules, CA

75 cm² Cell Culture Flask
Corning Inc., Corning, NY

Compressed Carbon Dioxide
Air Products, Pittsburgh, PA

Corning 6 Well Tissue Culture Plate
Corning Inc., Corning, NY

Corning 12 Well Tissue Culture Plate
Corning Inc., Corning, NY

Corning 96 Well Cell Culture Cluster Plate
Corning Inc., Corning, NY

Correlate-EIA Direct Cyclic AMP Kit
Assay Designs, Ann Arbor, MI

Cryogenic Vials
Nalgene, Rochester, NY

DAPI Stain
Invitrogen, Carlsbad, CA

Disposable Pasteur Pipettes
Fisher Scientific, Pittsburgh, PA

ECL-Plus
Amersham Arlington Heights, IL

Fisherbrand Redi-Tips™ Disposable Pipet Tips (1, 10, 200 µl)
Fisher Scientific, Pittsburgh, PA

Glass fiber filters (0.22 µm)
Schleicher & Schuell, Keene NH

Glass Microscope Slides
Fisher Scientific, Pittsburgh, PA

22x22 mm Glass Coverslips
Fisher Scientific, Pittsburgh, PA

Hemocytometer
American Optical Co., Buffalo, NY

Immobilon Transfer Membranes, Lot No. K2MN4305S
Millipore Co., Bedford, MA

Labsystem Adjustable Finnpiquette, D39077 (1-5 ml)
Fisher Scientific, Pittsburgh, PA

Labsystem Adjustable Finnpiquette, C99511 (200-1000 µl)
Fisher Scientific, Pittsburgh, PA

Labsystem Adjustable Finnpiquette, E08481 (40-200 µl)
Fisher Scientific, Pittsburgh, PA

Labsystem Adjustable Finnpiquette, D33981 (5-40 µl)

Fisher Scientific, Pittsburgh, PA

LIVE/DEAD[®] Viability/Cytotoxicity Kit
Molecular Probes, Eugene, OR

Microfuge Tubes, (1.5 µl)
Fisher Scientific, Pittsburgh, PA

Ready Gels, Lot No. L101105G2
BioRad Laboratories, Hercules, CA

Sigma Diagnostics Alkaline Phosphatase Kit
Sigma Chemical Co., St. Louis, MO

Sterile Disposable Serological Pipets (5, 10, 25, 50 ml)
Fisher Scientific, Pittsburgh, PA

Sterile Polystyrene Conical Tubes (15, 50 ml)
Becton-Dickinson, Lincoln Park, NJ

5. Equipment

Analytical Balance
Mettler-Toledo Inc., Hightstown, NJ

BioRad Power Pac 3000
BioRad, Hercules, CA

Cell Harvester
Brandel Biomedical R&D Labs, Inc., Gaithersburg, MD

Centrifuge Model 225 Centrifuge
Fisher Scientific, Pittsburgh, PA

Centrifuge Model 228 Centrifuge
Fisher Scientific, Pittsburgh, PA

Cobra II Auto-Gamma Counter
Packard Instrument Co., Downers Grove, IL

Drummond Pipet Aid
Drummond Scientific Co., Broomall, PA

Gel Apparatus
BioRad, Hercules, CA

Hot Plate with Stirrer
Fisher Scientific, Pittsburgh, PA

HTS 7000 Bio Assay Reader
Perkin Elmer, Norwalk, CT

Isotemp Incubator
Fisher Scientific, Pittsburgh, PA

Kodak M35A X-OMAT Processor
Kodak, New Haven, CT

Laminar Flow Hood
Forma Scientific Inc., Marietta, OH

Nikon Eclipse E600 microscope equipped with fluorescence optics
Fryer Co., Inc., Cleveland OH

Olympus CK2 Light Microscope
Olympus, Melville, NY

pH Meter 220
Corning Inc., Corning NY

Scion Series 7 Image Capture System
Scion Corporation, Frederick, MD

Tissue Tearor Variable Speed
Dremel, Racine, WI

Ultracentrifuge, Model L3-50
Beckman Instruments, Inc., Palo Alto, CA

UV/VIS Spectrophotometer, Model 6405
Jenway, Essex, UK

Water-Jacketed Incubator
Forma Scientific Inc., Marietta, OH

Weighing Balance
Denver Instrument, Denver, CO

6. Computer Software

Adobe Photoshop Version 5.0

Adobe Systems, Seattle, WA

AssayZap Version 2.51
Biosoft, Ferguson, MO

GraphPad Prism 3.0
GraphPad, San Diego, CA

Microsoft Excel, Microsoft Office 2003
Microsoft Corporation, Orem, UT

Microsoft Word, Microsoft Office 2003
Microsoft Corporation, Orem, UT

NIH-Image Version 1.62
Bethesda, MD

B. Methodology and Procedures

1. Cells and Cell Culture

Multipotent human adult mesenchymal stem cells (hAMSCs) capable of differentiating into osteoblasts [84 - 87] were used to evaluate melatonin's ability to enhance osteoblast differentiation. Human adult mesenchymal stem cells were purchased from Cambrex (Walkersville, MD) and maintained at 37°C, in 5% CO₂, and 90% humidity. Cells were passaged at 80% confluence. Cells (passage 5-10) were then seeded at a initial density of 3×10^3 cells/cm² [85] in 6 or 12 well plates, or T-75 plates in basal growth medium (Cambrex, Walkersville, MD), or osteogenic medium (Cambrex, Walkersville, MD) containing ascorbate, dexamethasone, and β -glycerophosphate for the indicated times.

2. Treatment Groups

Four treatment groups (OS-M-, OS-M+, OS+M-, OS+M+) were used throughout this study to analyze melatonin's effect on hAMSC differentiation into osteoblasts. Two of the treatment groups consisted of basal growth medium (OS-) containing either 0.05% ethanol vehicle (OS-M-) or 50 nM melatonin (OS-M+) (Sigma, St. Louis, MO). The basal growth medium was intended to promote hAMSC proliferation but not induce differentiation into an osteoblast. Treatments lasted for 10 days and were conducted to determine if melatonin could stimulate osteoblast differentiation independently from osteogenic medium. The remaining two groups (OS+M-, OS+M+) were exposed to osteogenic medium (OS+) in the absence (OS+M-) or presence (OS+M+) of melatonin to promote osteoblast

differentiation. These studies were conducted for 10 days to determine if melatonin could modulate osteogenic induced differentiation of hAMSCs into osteoblasts.

3. Melatonin Binding Assays

To determine whether or not melatonin binding sites were modified following hAMSC differentiation into an osteoblast, 2-[¹²⁵I]-iodomelatonin saturation analysis was performed as described previously [64]. Cells were seeded at a density of 3×10^3 cells/cm² in T-75 flasks and exposed for 5 or 10 days to (OS-M-), (OS-M+), (OS+M-), (OS+M+). Next, cells were washed five times with 10 ml of phosphate-buffered saline (PBS) (Invitrogen, Grand Island, NY) without calcium chloride or magnesium chloride, lifted from the plates using 10 mM KPO₄, 1 mM EDTA at 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-[¹²⁵I]-iodomelatonin (2,200 Ci/mmol; 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 within a treatment group and then averaged. The average K_D and B_{max} values were then compared between treatment groups. Statistics were performed using one-way ANOVA followed by a Newman-Keuls post hoc t-test where significance was set at $p < 0.05$.

4. Cyclic AMP Measurement

To determine whether melatonin receptor function changed following hAMSC differentiation, cAMP assays were performed using a direct cyclic AMP enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI). Briefly, hAMSCs were plated at a density of 3×10^3 cells/cm² and exposed to (OS-M-), (OS-M+), (OS+M-), (OS+M+) for 10 days. Next, cells were washed and then exposed to basal growth medium (no serum) containing rolipram (30 μ M) and/or forskolin (100 μ M) and appropriate concentrations of melatonin (1 fM – 1 μ M) for 10 min. Concentration-response curves were generated using Prism software. Potency (IC_{50}) and efficacy (maximum inhibition) values were obtained from each individual curve within a treatment group and then averaged. The average IC_{50} and maximum inhibition values were calculated for each treatment group and then compared between groups. Statistics were performed using an unpaired t-test where significance was set at $p < 0.05$.

5. MTT Assay

To assess whether melatonin and osteogenic medium affected hAMSC proliferation, cellular proliferation was assessed using 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue (MTT, Sigma, St. Louis, MO) assay after 7-day exposure to (OS-M-), (OS-M+), (OS+M-), (OS+M+). This time point was chosen to ensure that over-confluence of cells did not obscure the proliferation results. The MTT assay is dependent on cellular reduction of MTT by the mitochondrial dehydrogenase in living cells producing a formazan product that represents the number of living cells. The amount of formazan product produced from the MTT assay was solubilized using 0.1 N HCl in absolute isopropanol and analyzed spectrophotometrically at 595 nm. Statistics were performed using one-way ANOVA followed by a Newman-Keuls post hoc t-test where significance was set at $p < 0.05$.

6. Alkaline Phosphatase Assay

To assess whether melatonin modulated alkaline phosphatase (ALP) activity, a marker of osteoblast differentiation, ALP levels were measured by analyzing the rate of p-nitrophenyl phosphate disodium hexahydrate (pNPP) hydrolysis. Briefly, hAMSCs were plated at a density of 3×10^3 cells/cm² and exposed to (OS-M-) or (OS+M-) for 0, 5, or 10 days or (OS-M-), (OS-M+), (OS+M-), (OS+M+) for 10 days. ALP levels were normalized against cellular protein, determined via Pierce BSA assay (Pierce, Rockford, IL). The pNPP product was generated using a p-nitrophenol phosphate stock (Sigma, St. Louis,

MO) and analyzed according to the manufacturer's instructions to produce a soluble yellow end product that was analyzed spectrophotometrically. The pNPP reaction was then terminated using 1.0 M NaOH and immediately analyzed at 405 nm on a microplate reader and compared against a standard curve.

Alkaline phosphatase histochemical stain (Sigma, St. Louis, MO) was used to qualitatively detect the presence of alkaline phosphatase activity. Sample wells were washed three times with PBS, fixed in 80% methanol, 20% citrate working solution for one min, and then washed four times with distilled water. Alkaline phosphatase histochemical stain was then added, with incubation in the dark at room temperature for 1 h. Following staining, the samples were washed three times with PBS and viewed with bright field optics.

The involvement of melatonin receptors was assessed using pertussis toxin (60 ng/ml; Sigma, St. Louis, MO), and the melatonin receptor antagonists, luzindole (1 μ M; Tocris, Ballwin, MO) and 4P-PDOT (1 μ M; Tocris). EGFR involvement was assessed using 1.4 nM of an EGFR inhibitor, PD168393 (Calbiochem, La Jolla, CA). The role of MEK 1/2 and clathrin-mediated endocytosis were assessed using 15 μ M of PD98059, a MEK inhibitor (Calbiochem, La Jolla, CA) and 100 μ M monodansyl cadaverine (MDC) (Sigma, St. Louis, MO). PKA and the involvement of metalloproteinases were assessed using KT5720 (60 nM; Sigma) and GM 6001 (27 nM; Calbiochem), respectively. All of these antagonists or inhibitors were added to each of the treatment conditions for 10 days and ALP activity assessed as described previously. 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. Viability Staining

To ensure that melatonin or the various drugs were not cytotoxic to hAMSCs, Live/Dead assays (Molecular Probes, Eugene, OR) were performed for each treatment group according to the manufacturer's instructions. Live cells are distinguished from non-living cells through their esterase activity that converts nonfluorescent cell-permeable calcein AM to a green fluorescent calcein. Conversely, non-living cells are identified through an ethidium homodimer-1 dye, which enters damaged cell membranes and binds to nucleic acids, causing a red fluorescence that was visualized using a fluorescent microscope. The ethidium homodimer-1 dye is unable to pass through intact plasma membranes within living cells. Briefly, hAMSCs were plated at a density of 3×10^3 cells/cm² and exposed to (OS-M-), (OS-M+), (OS+M-), (OS+M+) for 10 days. Cell viability was then assessed as the percent of viable cells relative to the total population.

8. Western Blot Analysis

The effect of melatonin on modulating MEK/ERK (1/2) and EGFR activation during these differentiation processes was assessed using Western blot analysis. Briefly, hAMSCs were seeded at a density of 3×10^3 cells/cm² in T-75 flasks. One day after seeding, the cells were washed in sterile PBS and then incubated with 10 ml of either (OS-M-), (OS-M+), (OS+M-), or (OS+M+) treatment for 10 days. After the 10 day exposure, the medium was aspirated and

the cells were then washed with 10 ml of PBS. To the monolayer, 250 μ l of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 1 mM EGTA, and 1X protease inhibitors) were added. The resulting lysates were collected by scraping and then stored at -20°C until further use.

A BioRad DC protein assay (Bio-Rad, Hercules, CA) was performed on all samples to ensure equal loading of protein into each well of a 4-20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA). After electrophoresis at 200 V, the proteins were transferred to PVDF membranes (Millipore, Bedford, MA) presoaked in methanol and blocked in 5% nonfat dry milk dissolved in Tris-buffered Saline (25 mM Tris, pH 7.4, 140 mM NaCl, 2.7 mM KCl) for 1 h at room temperature. Membranes were incubated overnight at 4°C with anti-MEK (1/2) (1:10,000; Sigma, St. Louis, MO), anti-MEK-phos (1/2) (1:1,000; Cell Signaling, Beverly, MA), anti-ERK (1/2) (1:10,000; Sigma, St. Louis, MO), or anti-ERK-phos (1:1,000; Cell Signaling, Beverly, MA). For EGFR activation, mouse anti-EGFR (1 $\mu\text{g}/\mu\text{l}$; Calbiochem) was used. Following three washes in PBS, the membranes were then exposed to either anti-mouse (EGFR) or anti-rabbit IgG-HRP-linked secondary antibodies (1:2,000; Amersham Biosciences, Piscataway, NJ) for 1 h. Unbound secondary antibody was washed away with PBS, and the blots were developed using ECL-PLUS (Amersham Biosciences) for 5 min to visualize the protein bands. The blots were exposed to Kodak BioMax Light film (Fisher Scientific), developed using a Kodak M35A X-OMAT processor, and quantified using NIH Image densitometry software. All data were normalized against cells incubated with basal growth medium and vehicle (OS-M-) or

osteogenic medium and vehicle (OS+M-) and analyzed for statistical significance ($p < 0.05$) by an unpaired t-test or by one-way ANOVA followed by a Newman-Keul post hoc t-test.

9. Nuclear/Cytosol Fractionation

Nuclear and cytosolic fractions were separated as previously described [93]. Following vehicle or melatonin treatment, cells were washed with 1 x PBS and scraped into 500 μ l of lysis buffer [50 mM β glycerophosphate, 1 mM DTT, 1 mM EDTA, 1.5 mM EGTA, 1 mM Na_3VO_4 , containing 1 X complete mini protease inhibitors (Roche Diagnostics, Mannheim, Germany)]. The cells were then centrifuged (12,000 x g , 5 min, 4°C) and the resulting supernatant was discarded while the pellet was resuspended in 200 μ l of lysis buffer (40 mM β glycerophosphate, 1 mM DTT, 10 mM EGTA, 20 mM HEPES, 2 mM Na_3VO_4 , 1.0% Nonidet P-40). The lysate was mixed vigorously (10 sec) and then centrifuged (12,000 x g , 5 min, 4°C). The supernatant, representing the cytosolic fraction, was separated from the pellet which represented the nuclear fraction. To extract the nuclear proteins from the pellet, 100 μ l of nuclear extraction buffer (50 mM β glycerophosphate, 1 mM DTT, 200 μ M EDTA, 25% glycerol, 1.5 mM MgCl_2 , 420 mM NaCl, 500 μ M Na_3VO_4) was added to the pellet, sonicated (2 x 5 sec, 40 W, 4°C), vortexed (10 sec) and centrifuged (12,000 x g , 5 min, 4°C). The purity of the nuclear fraction was determined by DAPI staining (Invitrogen, Carlsbad, CA) for nucleic acids. To evaluate melatonin's effect on ERK translocation or activation (ERK-P) within the cytoplasm or nucleus, equal

amounts of protein were loaded onto a 4-20% gradient acrylamide gel and subjected to Western blot analysis. Total ERK and ERK-P levels were detected using anti-ERK (1:10,000, Sigma, St. Louis, MO) or anti-ERK-P (1:1,000, Cell Signaling, Beverly, MA) antibodies, respectively. Proteins were visualized by ECL and the bands were quantified by densitometry using NIH Image software. Each bar represents the mean \pm SEM of 5-6 experiments. All data were analyzed by one-way ANOVA followed by a Newman-Keuls post hoc t-test where significance was defined as $p < 0.05$

10. Immunoprecipitation

Cells were incubated with OS+ medium for 10 days. The medium was then aspirated and washed with 1X PBS. Lift buffer (4 ml) consisting of (10 mM KPO_4 , 1 mM EDTA at pH 7.4) was then added to each plate for 5 min and centrifuged (500 x *g*, 3 min). Five hundred microliters of buffer A (0.5 M EDTA, 12 mM $MgCl_2$, 1 M Tris containing 1 X complete mini protease inhibitors (Roche Diagnostics, Mannheim, Germany)) was added to the resulting pellet, homogenized (2 x 15 second, 30,000 rpm, 4°C) and centrifuged (1,800 x *g*, 30 min). A 1% digitonin (Sigma, St. Louis, MO) solution was added (495 μ l) to the resulting pellet and resuspended. Five microliters of vehicle (0.05% ethanol), 0.1 μ M melatonin, or 0.1 μ M melatonin + 100.0 μ M luzindole were added to separate reactions, vortexed, and incubated at 4°C for 3 h. The solutions were then centrifuged (12,000 x *g*, 5 min). Buffer A (495 μ l) and 5 μ l of vehicle, 0.1 μ M melatonin, or 0.1 μ M melatonin + 100.0 μ M luzindole were added to the resulting

supernatants and vortexed. Ten microliters of rabbit anti-human MT₁ or MT₂ melatonin receptor primary antibody (1:2,000) was then added to each solution and rocked at 4°C for 12 h. Protein A agarose beads (50 µl) (Sigma, St. Louis, MO) were added to the solutions and incubated at 4°C for an additional 6 h. Solutions were then centrifuged (12,000 x *g*, 5 min). Ten microliters of 100 µM GppNHp (Sigma, St. Louis, MO) and an additional 50 µl protein A agarose beads were added to each solution, vortexed, and incubated at 37°C for 1 h to uncouple the receptors from their G-proteins. The solutions were then centrifuged (12,000 x *g*, 1 min). The supernatant, containing the proteins associated with each of the melatonin receptors, and the pellet, containing the melatonin receptor IgG complex were separated. Fifteen percent trichloroacetic acid (Sigma, St. Louis, MO) was added to the supernatant fraction to precipitate out the proteins in the cytosol and incubated at 4°C for 5 min. The supernatant was then centrifuged (12,000 x *g*, 1 min) and an equal volume of SDS sample buffer (0.2% bromophenol blue, 200 mM DTT, 20% glycerol, 4% SDS, 100 mM Tris Cl) was added to the resulting pellet. Pellets were subjected to electrophoresis and Western blot as described to visualize the MT₁ or MT₂ melatonin receptor, G_{αi-2} (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), β-arrestin-1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), β-arrestin-2 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), EGFR (1:500, Lab Vision, Fremont, CA), MEK (1:10,000 Sigma, St. Louis, MO) or ERK (1:10,000 Sigma, St. Louis, MO). Each protein band was normalized against the total amount of melatonin receptor immunoprecipitated. All data were analyzed by one-way ANOVA followed by a

Newman-Keuls post hoc t-test where significance was defined as $p < 0.05$.

III. RESULTS AND DISCUSSION

A. Results

1. *The effect of osteogenic medium on ALP activity in hAMSCs*

ALP activity was measured as a marker for hAMSC differentiation into osteoblasts. A time-dependent effect of osteogenic medium on ALP activity was observed. ALP activity was detected by day 5 of (OS+)-treatment that reached significance by day 10 (Fig. 1). The 10-day time point was thus chosen because it was at this point where significant increases in ALP activity occurred and prior to the deposition of significant amounts of calcium [84].

2. *The effect of melatonin and osteogenic medium on ALP activity in hAMSCs*

As shown in figure 2, ALP activity increased in hAMSCs following a 10-day incubation in osteogenic medium supplemented with vehicle (OS+M-) or melatonin (OS+M+) when compared to cells exposed to growth medium containing vehicle (OS-M-) or melatonin (OS-M+). Melatonin (50 nM), added to the basal growth medium (OS-M+), did not significantly increase ALP activity compared to cells exposed to vehicle (OS-M-) alone. However, when hAMSCs were exposed to melatonin, in combination with the osteogenic medium (OS+M+), ALP activity significantly ($p < 0.05$) increased beyond that of cells exposed to osteogenic medium alone (OS+M-). These data indicate that melatonin enhances ALP activity in hAMSCs when osteogenic medium is present (Fig. 2).

Figure 1: The effect of osteogenic medium on ALP activity in hAMSCs.

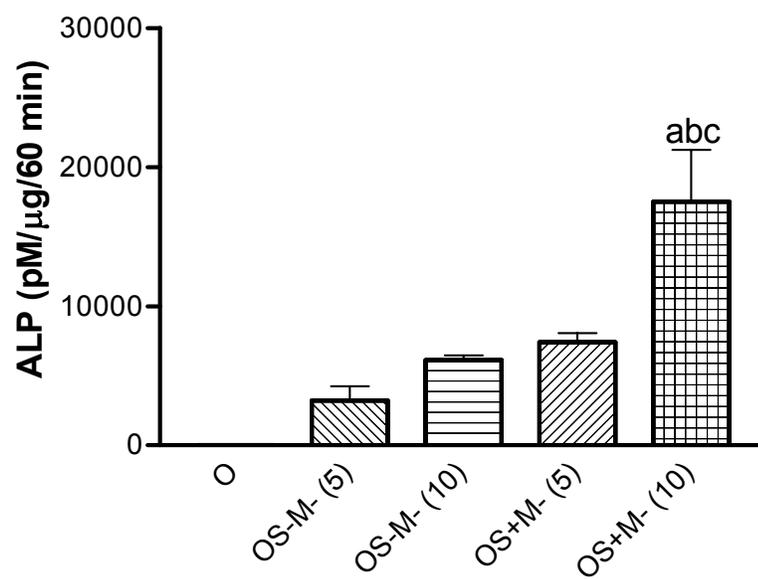


Figure 1: The effect of osteogenic medium on ALP activity in hAMSCs.

Cells were seeded at a density of 3000 cells/cm² in 12-well dishes and cultured in growth medium (OS-) in the absence of melatonin (OS-M-) or in osteogenic medium (OS+) in the absence of melatonin (OS+M-) for 0, 5, or 10 days. Following the exposure period, cells were assayed for ALP activity. As shown, there was a significant increase in ALP activity when cells were exposed to osteogenic medium (OS+M-) for 10 days compared to cells cultured in growth medium (OS-M-) for 5 or 10 days. Additionally, there was a significant increase in ALP activity in cells exposed to OS+M- treatment for 10 days compared to 5 days. Each bar represents the mean \pm SEM of 3 experiments. All data were analyzed by one-way ANOVA ($F = 12.81$; 4, 9 df; $p < 0.001$) followed by a Newman-Keuls post hoc t-test where significance was defined as $p < 0.05$, where a = $p < 0.05$ when compared to OS-M- (5); b = $p < 0.05$ when compared to OS-M- (10); c = $P < 0.05$ when compared to OS+M- (5).

Figure 2: The effect of melatonin and osteogenic medium on ALP activity in hAMSCs.

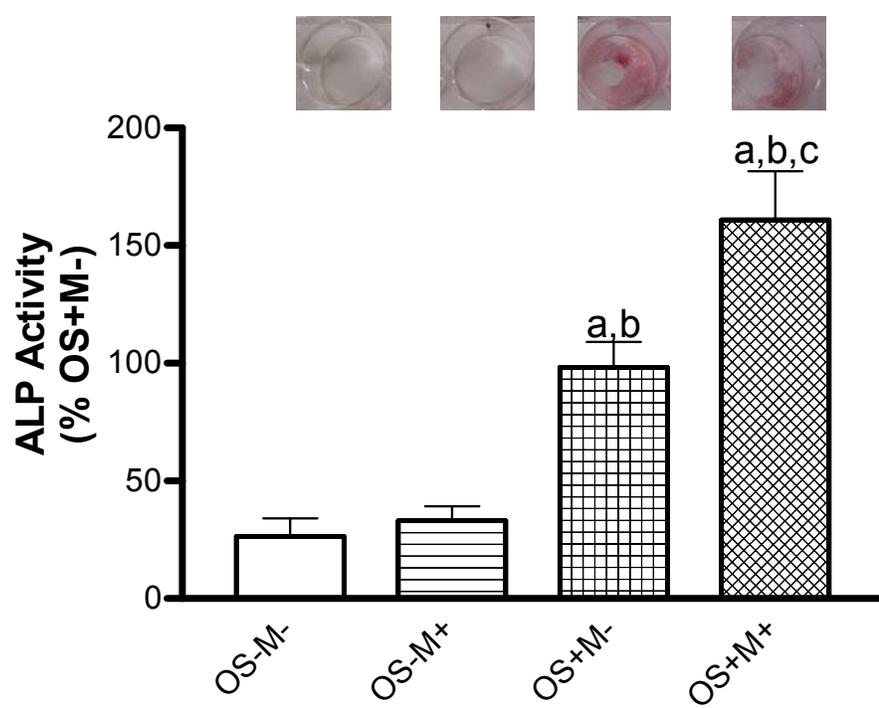


Figure 2: The effect of melatonin and osteogenic medium on ALP activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² in 12-well dishes and cultured in either growth medium (OS-) in the absence (OS-M-) or presence of melatonin (OS-M+) or in osteogenic medium (OS+) in the absence (OS+M-) or presence of melatonin (OS+M+) for 10 days. Following the 10- day exposure, cells were assayed for ALP activity. As shown, there was a significant increase in ALP activity when cells were exposed to osteogenic medium alone (OS+M-) compared to cells cultured in growth medium (OS-M-). Melatonin, added in combination with the osteogenic medium (OS+M+), enhanced further ALP activity compared to vehicle-exposed cells. Melatonin had no effect when added to the growth medium alone. Each bar represents the mean \pm SEM of 14 experiments performed in duplicate. All data were analyzed by one-way ANOVA (F = 24.75; 3, 52 df; p < 0.0001) followed by a Newman-Keuls post hoc t-test where significance was defined as p < 0.05 where a = p < 0.05 when compared to OS-M-; b = p < 0.05 when compared to OS-M+; c = P < 0.05 when compared to OS+M-. [INSET: Representative photomicrographs of hAMSCs exposed to each treatment and then stained with alkaline phosphatase histochemical stain after 10 days to visualize ALP activity. As shown, the intensity of staining increased in cells cultured in osteogenic medium when compared to cells cultured in growth medium alone].

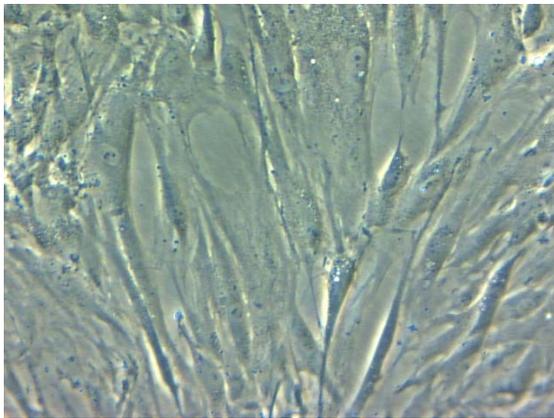
3. The effect of melatonin and osteogenic medium on hAMSCs morphology

To further evaluate osteoblast differentiation, the effect of melatonin and osteogenic medium on hAMSC morphology was evaluated. A striated morphology, characteristic of an undifferentiated hAMSC, was uniformly observed in cells incubated with either vehicle (OS-M-) or melatonin (OS-M+) supplemented to the OS- medium (Figs. 3A & 3B). This result corresponds with the biochemical ALP assay, as melatonin was unable to independently induce osteoblast morphology in hAMSCs following 10-day incubation. Conversely, hAMSCs incubated with OS+ medium supplemented with either vehicle (OS+M-) or melatonin (OS+M+) induced a cuboidal morphology characteristic of a differentiated osteoblast (Figs. 3C & 3D). An additive effect between melatonin and osteogenic medium could not be assessed, as the cuboidal morphology was overly apparent in both OS+M- and OS+M+ incubated cells.

4. The effect of pertussis toxin, luzindole, or 4P-PDOT on ALP activity in hAMSCs

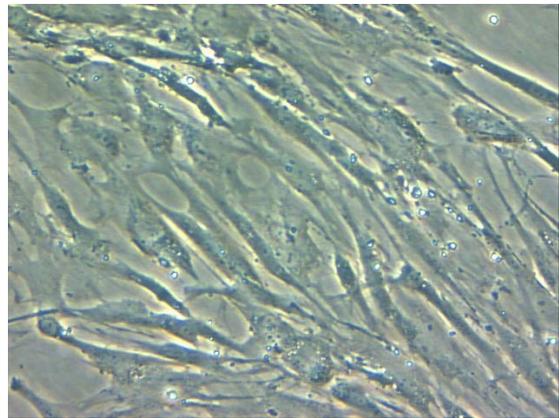
To assess whether these effects of melatonin on ALP activity were mediated through melatonin receptors, pertussis toxin, and the non-specific melatonin receptor antagonist, luzindole and MT₂-selective melatonin receptor antagonist 4P-PDOT, were added to the (OS+M-) and (OS+M+) treatments. Pertussis toxin (60 ng/ml), luzindole (1 μ M) [94] and 4P-PDOT (1 μ M) [95] alone were without effect on inducing ALP activity over that induced by OS+ (Table 1).

Figure 3: The effect of melatonin and osteogenic medium on hAMSCs morphology.



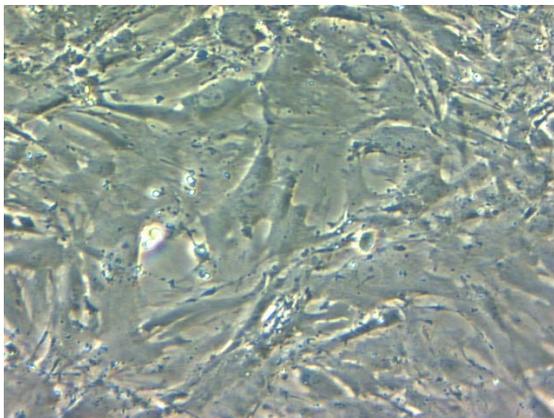
A.

OS-M-



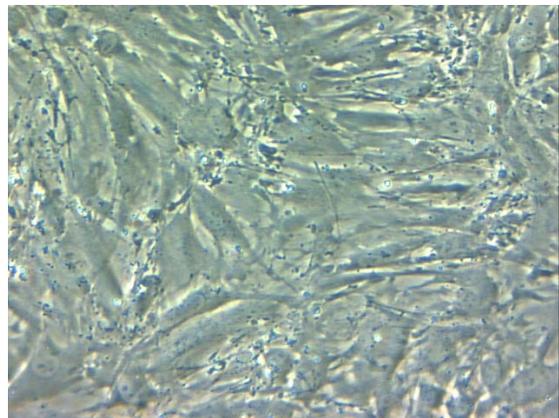
B.

OS-M+



C.

OS+M-



D.

OS+M+

Figure 3: The effect of melatonin and osteogenic medium on hAMSCs morphology. Cells were seeded at a density of 3000 cells/cm² in T-75 flasks and then cultured in either growth medium (OS-) in the absence (OS-M-, A.) or presence (OS-M+, B.) of melatonin or in osteogenic medium (OS+) in the absence (OS+M-, C.) or presence (OS+M+, D.) of melatonin for 10 days. Following the 10- day exposure, morphology was assessed at 100X total magnification. As shown, morphology of hAMSCs incubated in OS- medium with either vehicle or melatonin appeared striated, characteristic of an undifferentiated stem cell, indicating melatonin is unable to independently induce osteoblast morphology. Conversely, hAMSCs incubated in OS+ medium in either vehicle or melatonin induced a cuboidal morphology characteristic of a differentiated osteoblast.

Table 1. The effect of melatonin receptor antagonists and various inhibitors on ALP activity in hAMSCs incubated in osteogenic medium containing vehicle (OS+M-) or melatonin (OS+M+).

Treatment	Average ALP Activity \pm SEM (% OS+M-)	
	<i>OS+M-</i>	<i>OS+M+</i>
<i>Vehicle</i>	98 \pm 11	-
<i>Melatonin</i>	-	161 \pm 21 ^a
+ <i>Pertussis toxin</i>	43 \pm 15	21 \pm 20 ^b
+ <i>Luzindole</i>	71 \pm 18	86 \pm 15 ^b
+ <i>4P-PDOT</i>	66 \pm 12	77 \pm 25 ^b
+ <i>MDC</i>	59 \pm 5	40 \pm 10 ^b
+ <i>PD98059 (MEK I)</i>	41 \pm 13	50 \pm 9 ^b
+ <i>PD168393 (EGFR I)</i>	69 \pm 16	87 \pm 36 ^b

Table 1. The effect of melatonin receptor antagonists and various inhibitors on ALP activity in hAMSCs incubated in osteogenic medium containing vehicle (OS+M-) or melatonin (OS+M+). Data represent the mean \pm SEM of 4-14 experiments performed in triplicate. All data were analyzed by one-way ANOVA followed by a Newman-Keuls post hoc t-test where significance was defined as $p < 0.05$, where ^a = $p < 0.05$ when compared to (OS+M-)-treated cells; ^b = $p < 0.05$ when compared to (OS+M+)-treated cells.

However, the melatonin-mediated increase in ALP activity was blocked in the presence of these agents, indicating that melatonin enhanced ALP activity through melatonin receptors, and most probably, the MT₂ melatonin receptor (Fig. 4).

To determine whether melatonin receptors were present in hAMSCs, saturation binding analysis was performed using the agonist radioligand 2-[¹²⁵I]-iodomelatonin. Following exposure to basal growth medium (OS-M-) or to osteogenic medium (OS+M-), 2-[¹²⁵I]-iodomelatonin binding sites were detected and found not to be significantly different from one another [(OS-M-): K_D = 27 pM range of SEM (17-45 pM); B_{max} = 2.5 ± 1.0 fmol/mg/protein; (OS+M-): K_D = 30 pM range of SEM (26-34 pM); B_{max} = 3.3 ± 0.4 fmol/mg/protein, n = 5]. However, chronic 10-day exposure of hAMSCs to melatonin, in either OS- or OS+ medium, resulted in a total loss of detectable specific 2-[¹²⁵I]-iodomelatonin binding sites.

To assess whether functional melatonin receptors were present in hAMSCs, cAMP accumulation assays were performed. Concomitant with the appearance of 2-[¹²⁵I]-iodomelatonin binding sites in hAMSCs incubated in OS+M- medium, functional melatonin receptors were detected as well. A concentration-dependent decrease in cAMP formation occurred with a potency value [IC₅₀ = 0.07 pM range of SEM (0.02-0.2 pM), efficacy at 1 pM melatonin = 66 ± 4% inhibition), n = 4]. However, hAMSCs incubated in osteogenic medium and melatonin (OS+M+) showed no concentration-dependent inhibition of cAMP (efficacy at 1 pM melatonin = 12 ± 7% inhibition, n = 4). These results correlate

Figure 4: The effect of pertussis toxin, luzindole, or 4P-PDOT on ALP activity in hAMSCs.

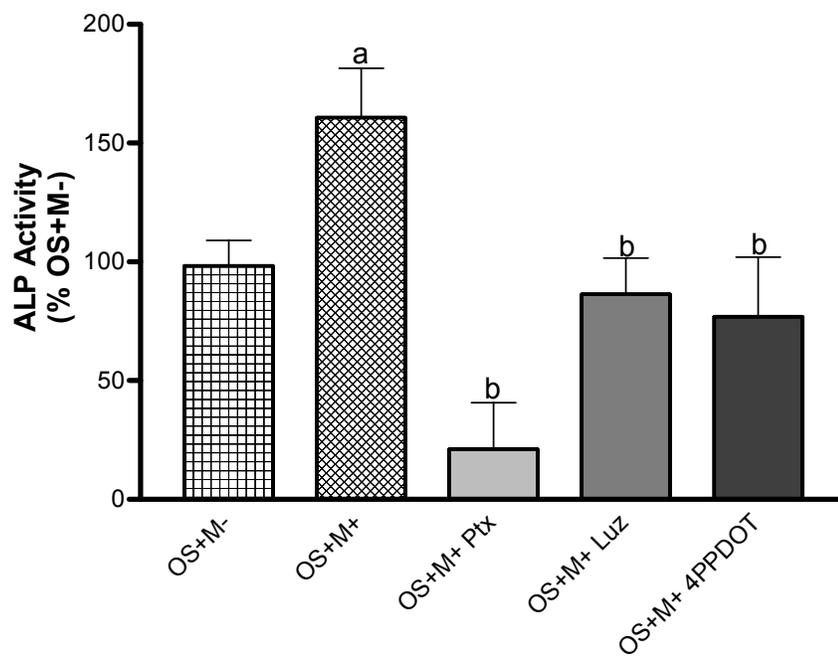


Figure 4: The effect of pertussis toxin, luzindole, or 4P-PDOT on ALP activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² in 12-well culture dishes and then cultured in melatonin-supplemented osteogenic medium (OS+M+) in the absence or presence of pertussis toxin (60 ng/ml), 4P-PDOT (1 μ M), or luzindole (1 μ M). Following a 10-day exposure, cells were assayed for ALP activity. As shown, pertussis toxin blocked the enhancing effects of melatonin indicating the involvement of Gi/Go proteins. Furthermore, the melatonin receptor antagonists, luzindole and 4P-PDOT, also blocked these effects indicating the involvement of melatonin receptors, in particular, MT₂ melatonin receptors. Each bar represents the mean \pm SEM of 4-14 experiments performed in duplicate. All data were analyzed by one-way ANOVA (F = 5.81; 4, 40 df; p < 0.001) followed by a Newman-Keuls post hoc t-test where significance was defined as p < 0.05, where a = p < 0.05 when compared to OS+M-, b = p < 0.05 when compared to OS+M+. Abbreviations: Ptx = pertussis toxin; Luz = luzindole.

with the lack of detectable specific 2-[¹²⁵I]-iodomelatonin binding revealed by the saturation analysis. These data suggest that desensitization of melatonin receptors occurred in hAMSCs exposed chronically to melatonin.

5. The effect of melatonin and/or osteogenic medium on hAMSC proliferation

The effects of these various treatments on cellular proliferation of hAMSC were assessed because melatonin has been shown to reduce proliferation in a variety of cells types [96-104] and also because an attenuation of proliferation is usually associated with differentiated cell types [105, 106]. As shown in figure 5, melatonin or osteogenic medium alone inhibited proliferation by 11-14% ($p < 0.05$) when compared to hAMSCs incubated in growth medium (OS-M-). Melatonin, added to the osteogenic medium, did not further enhance these inhibitory effects.

Figure 5: The effect of melatonin and/or osteogenic medium on hAMSC proliferation.

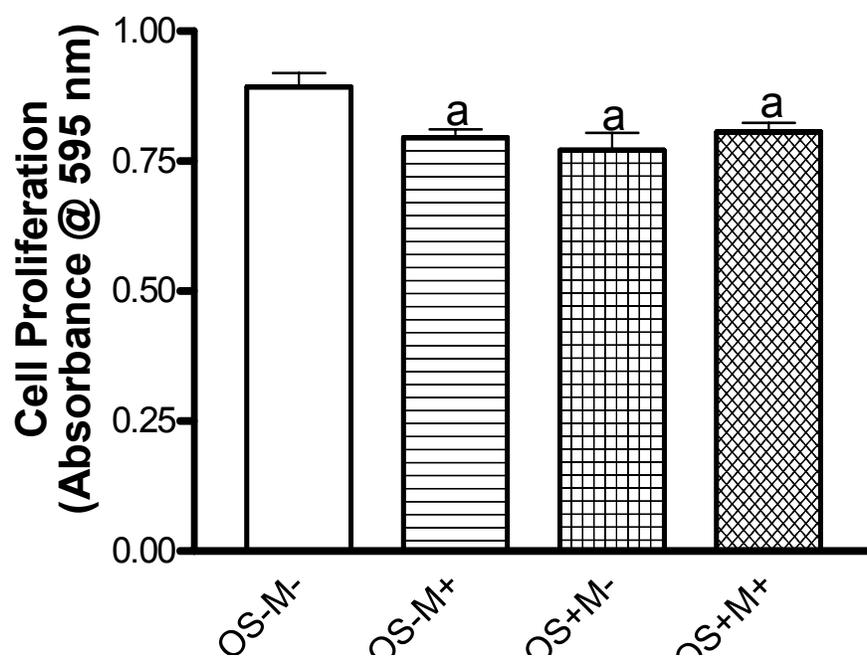


Figure 5: The effect of melatonin and/or osteogenic medium on hAMSC proliferation. Cells were seeded at a density of 3000 cells/cm² in 12-well dishes and cultured in either growth medium (OS-) in the absence (OS-M-) or presence of melatonin (OS-M+) or in osteogenic medium (OS+) in the absence (OS+M-) or presence of melatonin (OS+M+) for 7 days. After the seventh day of incubation, cellular proliferation was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue (MTT) assay. As shown, a subtle yet significant inhibition of cellular proliferation occurred following exposure to melatonin alone or to osteogenic medium supplemented with 50 nM melatonin. Each bar represents the mean \pm SEM of 5-6 experiments performed in quadruplicate. All data were analyzed by one-way ANOVA (F = 4.36; 3, 18 df; p < 0.05) followed by a Newman-Keuls post hoc t-test where significance was defined as P < 0.05 where a = p < 0.05 when compared to OS-M-.

6. The effect of a MEK inhibitor, PD98059, an EGF receptor inhibitor, PD 168393 and (coated pit inhibitor) monodansyl cadaverine on ALP activity in hAMSCs

To investigate some potential mechanisms underlying MT₂ melatonin receptor-mediated effects on ALP activity, the receptor tyrosine kinase pathway and mitogen activated signaling pathways were targeted. Using selective inhibitors of each (1 nM of the selective MEK (1/2) inhibitor, PD98059, and, 1.4 nM of the EGFR inhibitor, PD168393), their effects on promoting or preventing ALP activity in differentiating hAMSCs were assessed. When added alone, these inhibitors had no effect when compared to cells exposed to osteogenic medium alone (OS+M-) (Table 1). However, when these inhibitors were added in combination with melatonin, they blocked the melatonin-induced increases in ALP activity (Fig. 6).

As proposed by Pierce and co-workers [107], MEK activation by G-protein coupled receptors (GPCRs) may be modulated by clathrin-mediated endocytosis. Monodansyl cadaverine (MDC), a clathrin-coated pit inhibitor, was added to the (OS+M-) and (OS+M+) treatments. As shown in figure 6 and Table 1, the addition of MDC (100 μM) prevented any of the increases in ALP activity induced by melatonin. These data implicate EGFRs, the MEK (1/2) signal transduction cascade, as well as clathrin-mediated endocytosis, in modulating melatonin's effect on ALP activity in hAMSCs.

Figure 6: The effect of a MEK inhibitor, PD98059, an EGF receptor inhibitor, PD 168393 and (coated pit inhibitor) monodansyl cadaverine on ALP activity in hAMSCs.

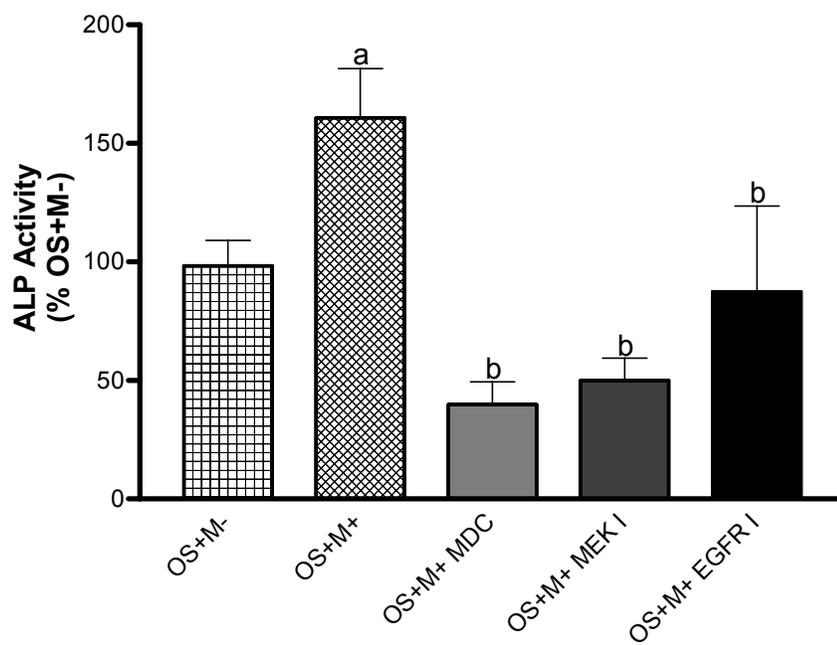


Figure 6: The effect of a MEK inhibitor, PD98059, an EGF receptor inhibitor, PD 168393 and (coated pit inhibitor) monodansyl cadaverine on ALP activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 12-well culture dishes and then cultured in melatonin-supplemented osteogenic medium (OS+M+) in presence of PD98059 (1 nM), PD 168393 (1.4 nM), or MDC (100 mM). Following a 10-day exposure, cells were assayed for ALP activity. As shown, all inhibitors blocked the enhancing effects of melatonin indicating the involvement of the MEK/ERK signaling cascade, EGF receptor activation and clathrin-mediated endocytosis in these processes. Each bar represents the mean \pm SEM of 4-14 experiments performed in duplicate. All data were analyzed by one-way ANOVA ($F = 6.11$; 4, 40 df; $p < 0.001$) followed by a Newman-Keuls post hoc t-test where significance was defined a $p < 0.05$, where a = $p < 0.05$ when compared to OS+M-, b = $p < 0.05$ when compared to OS+M+. Abbreviations: MDC = monodansyl cadaverine; MEK I = MEK inhibitor, (PD98059); EGFR I = EGF receptor inhibitor, (PD168393).

7. The effect of an acute exposure to melatonin on EGFR activity in hAMSCs

As shown in figure 6, co-incubation of an EGFR inhibitor with melatonin prevented any melatonin-induced enhancement of ALP activity. To determine if an acute exposure to melatonin alone activated EGFRs, hAMSCs that had already been exposed to osteogenic medium (OS+M-) for 10 days were rechallenged with 50 nM melatonin (OS+M-/M+). This treatment (OS+M-) previously resulted in high 2-[¹²⁵I]-iodomelatonin binding and high potency of melatonin to inhibit forskolin-induced cAMP accumulation. As shown in figure 7, melatonin exposure for 10 min resulted in a significant increase in EGFR activity when compared to cells rechallenged with vehicle alone (OS+M-/M-).

8. The effect of an acute exposure to melatonin on ERK activity in hAMSCs

An inhibition of the MEK/ERK (1/2) signal transduction pathway was sufficient to block the melatonin-induced osteoblast differentiation in hAMSCs (Fig. 6). To determine if an acute exposure to melatonin activated ERK, western blot analysis was performed in hAMSCs exposed to osteogenic medium alone (OS+M-). Again, it was this treatment (OS+M-) that resulted in high 2-[¹²⁵I]-iodomelatonin binding and high potency of melatonin to inhibit forskolin-induced cAMP accumulation. Chronic 10-day exposure of hAMSCs to osteogenic medium followed by a melatonin rechallenge (OS+M-/M+), resulted in an increase in ERK-P without changing total ERK (1/2) expression levels when compared to cells rechallenged with vehicle (OS+M-/M-) alone (Fig. 8).

Figure 7: The effect of an acute exposure to melatonin on EGFR activity in hAMSCs.

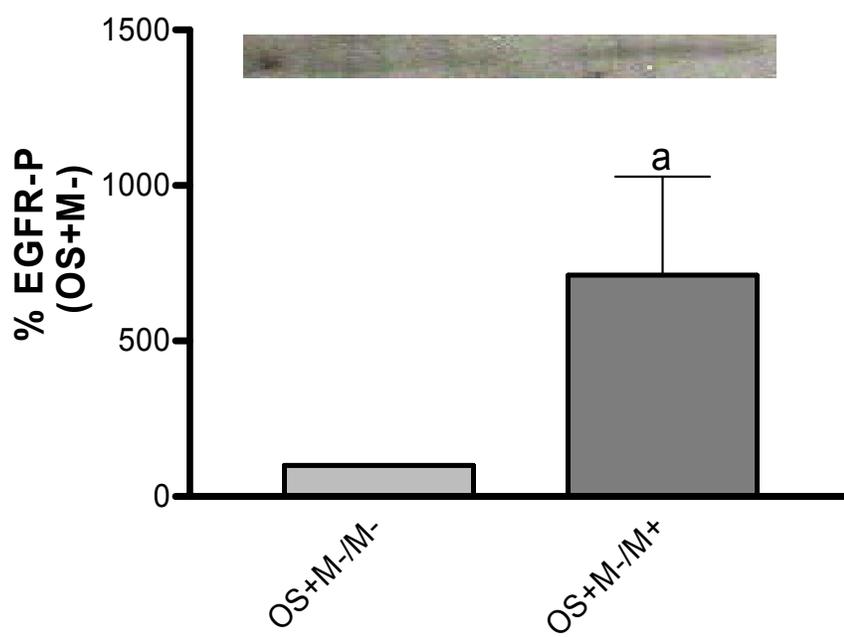


Figure 7: The effect of an acute exposure to melatonin on EGFR activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 10 cm culture dishes and then grown in osteogenic medium in the absence of melatonin (OS+M-) for 10 days. Following the 10- day exposure, cells were rechallenged with either vehicle (OS+M-/M-) or melatonin (OS+M-/M+) for 10 min and then lysed in sample buffer. EGFR phosphorylation was detected using mouse anti-EGFR (pTyr1173; 1 mg/ml) followed by anti-mouse IgG-HRP as discussed in the Methods. Proteins were visualized using ECL and the bands were quantified by densitometry using NIH Image software. The bands within an experiment were normalized against the OS+M-/M- controls. As shown, in cells cultured in osteogenic medium followed by an acute exposure to melatonin, a significant increase in EGFR-P occurred. Each bar represents the mean \pm SEM of 3 experiments. All data were analyzed by an unpaired t-test ($t = 2.44$; 6 df; $p < 0.05$) where significance $a = p < 0.05$ when compared to OS+M-/M- [INSET: A representative blot of EGFR-P].

Figure 8: The effect of an acute exposure to melatonin on ERK activity in hAMSCs.

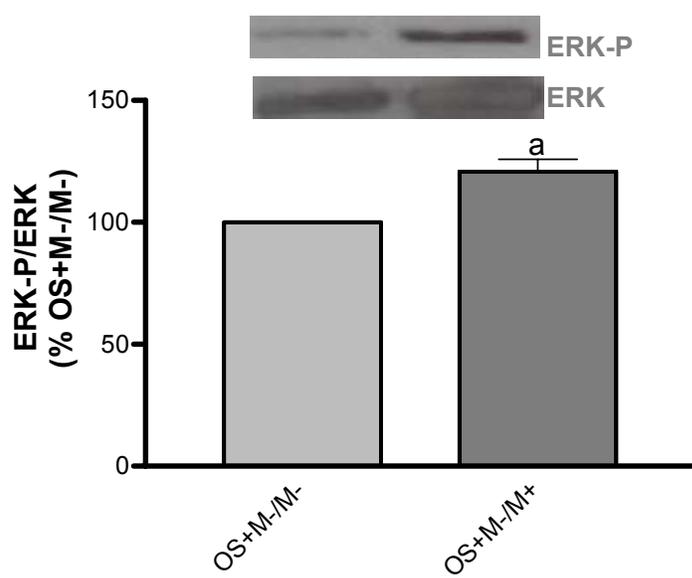


Figure 8: The effect of an acute exposure to melatonin on ERK activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 10 cm culture dishes and then grown in osteogenic medium in the absence of melatonin (OS+M-) for 10 days. Following the 10- day exposure, cells were rechallenged with either vehicle (OS+M-/M-) or melatonin (OS+M-/M+) for 10 min and then lysed in sample buffer. Equal amounts of protein were loaded onto a 4-20% gradient acrylamide gel and subjected to Western blot analysis. Total ERK and ERK-P levels were detected using anti-ERK (1:10,000) or anti-ERK-P (1:1,000) antibodies. Proteins were visualized using ECL and the bands were quantified by densitometry using NIH Image software. The bands within an experiment were normalized against the OS+M-/M- controls. As shown, in cells cultured in osteogenic medium followed by an acute exposure to melatonin, a significant increase in ERK-P occurred. No changes in total ERK levels occurred. Each bar represents the mean \pm SEM of 3 experiments. All data were analyzed by an unpaired t-test ($t = 2.80$; 9 df; $p < 0.05$) where significance $\alpha = p < 0.05$ when compared to OS+M-/M- [INSET: Representative blots of ERK-P (upper blot) and ERK (lower blot)].

Cytotoxicity analysis was performed on all treatment groups to ensure that the loss in ALP activity was not due to cell death. Using a live/dead cytotoxicity assay, the percent of viable cells relative to control (OS+M+) was unchanged [(OS+M-/Ptx) = 149%, (OS+M-/PD98059) = 168%, (OS+M-/MDC) = 103%, (OS+M-/PD168393) = 100%, (OS+M-/4P-PDOT) = 96%, and (OS+M-/luzindole) = 100%]. These data indicate that the concentrations of inhibitors or antagonists used were not cytotoxic to the hAMSCs.

9. The effect of melatonin and/or osteogenic medium on MEK or ERK activity in hAMSCs

Results from the ALP assays indicated that the MEK (1/2) signal transduction pathway was necessary for the melatonin-induced osteoblast differentiation. To assess the chronic effects of melatonin's exposure on MEK and ERK, western blot analysis was performed, analyzing total MEK and ERK levels as well as their activation states (i.e, MEK-P and ERK-P). Chronic 10-day exposure of hAMSCs to melatonin and osteogenic medium, resulted in a decrease in MEK-P and ERK-P without changing total MEK and ERK (1/2) expression levels when compared to (OS+M-)-treated cells. Melatonin, added to growth medium (OS-M+) was without effect on either the activity or expression levels of MEK and ERK (Fig. 9).

Figure 9: The effect of melatonin and/or osteogenic medium on MEK or ERK activity in hAMSCs.

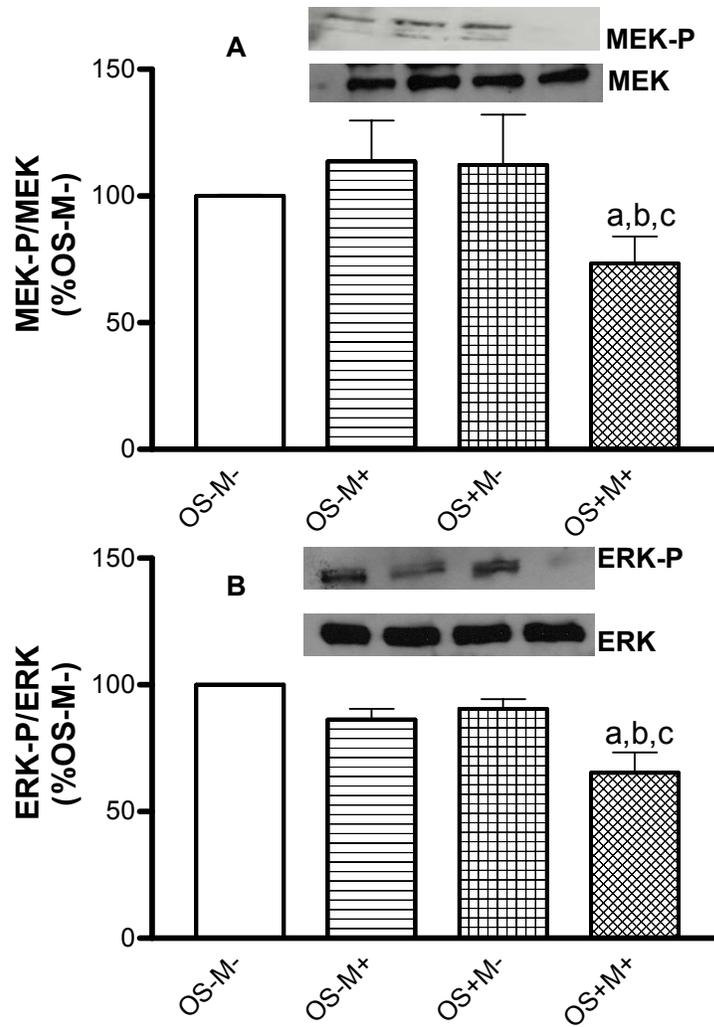


Figure 9: The effect of melatonin and/or osteogenic medium on MEK or ERK activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 10 cm culture dishes and then grown in either growth medium in the absence (OS-M-) or presence (OS-M+) of melatonin or to osteogenic medium in the absence (OS+M-) or presence (OS+M+) of melatonin for 10 days. Following the 10- day exposure, cells were lysed in sample buffer. Equal amounts of protein were loaded onto a 4-20% gradient acrylamide gel and subjected to western blot analysis. Total MEK and ERK levels were detected using anti-MEK (1:10,000) or anti-ERK (1:10,000) antibodies whereas MEK-P and ERK-P levels were detected using anti-MEK-P (1:1,000) or anti-ERK-P (1:1,000) antibodies. Proteins were visualized using ECL and the bands were quantified by densitometry using NIH Image software. The bands within an experiment were normalized against the OS-M- controls. As shown, in cells cultured in osteogenic medium supplemented with melatonin (OS+M+), a significant decrease in both MEK-P (A) and ERK-P (B) occurred without changes in total levels of these proteins. Each bar represents the mean \pm SEM of 3-6 experiments. All data were analyzed by one-way ANOVA ($F = 8.86; 3, 20 \text{ df}; p < 0.0001$) followed by a Newman-Keuls post hoc t-test where significance was defined as $p < 0.05$ where a = $p < 0.05$ when compared to OS-M-; b = $p < 0.05$ when compared to OS-M+ and c = $p < 0.05$ when compared to OS+M-. [INSET: Representative blots of MEK-P (upper blot in (A) and MEK lower blot in (A) and ERK-P (upper blot in (B) and ERK (lower blot in (B))].

10. The effects of KT5720, a PKA inhibitor, on ALP activity in hAMSCs

Melatonin receptors can couple to G_i proteins resulting in decreases in PKA [108, 109]. The role of PKA in modulating melatonin-induced increases in ALP activity was assessed using a PKA inhibitor, (PKI: KT5720). As shown in figure 10, co-incubation of KT5720 with melatonin and osteogenic medium (OS+M+/PKI) did not modulate the melatonin-induced increase in ALP activity when compared to (OS+M+)-treated groups. The inhibitor, when added alone (OS+M-/PKI) was not significantly different from (OS-M+)-treated groups.

11. The effects of GM 6001, a metalloproteinase inhibitor, on ALP activity in hAMSCs

The role of EGFRs in modulating melatonin-induced enhancement of ALP activity was further assessed using a metalloproteinase inhibitor, GM 6001. As shown in figure 11, co-incubation of this metalloproteinase inhibitor (MPI) with melatonin and osteogenic medium (OS+M+/MPI) blocked any melatonin-induced increase in ALP activity when compared to (OS+M+)-treated groups. In addition, this inhibitor, when added alone (OS+M-/MPI) was not significantly different from (OS+M-)-treated groups or from cells exposed to growth medium (OS-) alone. Thus, unlike the other inhibitors, GM 6001 treatment alone prevented (OS+M-)-induced increases in ALP activity. These data show that metalloproteinases were essential in ALP activity induced by osteogenic medium alone and perhaps melatonin. The role of specific metalloproteinases in modulating melatonin's enhancing effects on ALP activity in differentiating hAMSCs will be defined in future studies using selective MPIs.

Figure 10: The effect of KT5720, a PKA inhibitor, on ALP activity in hAMSCs.

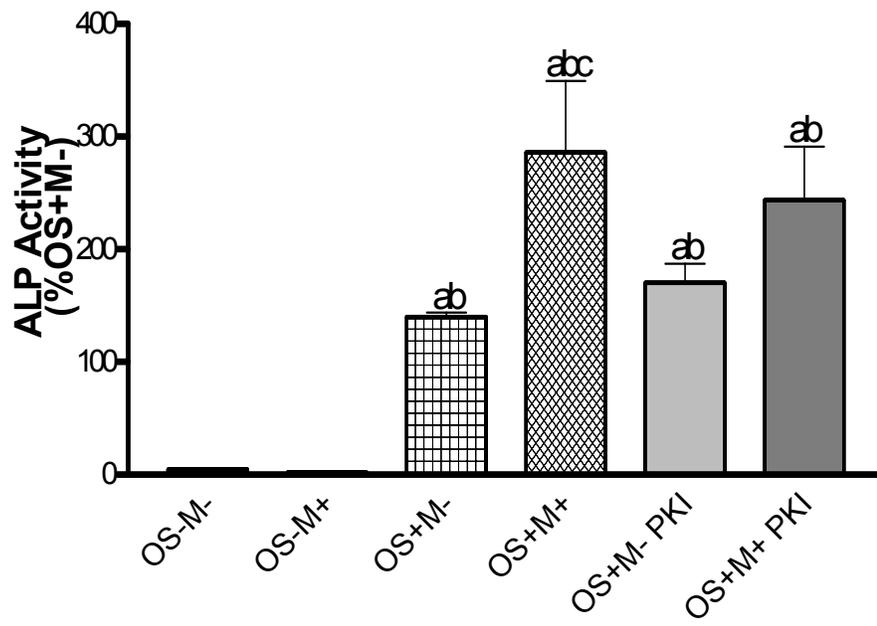


Figure 10: The effect of KT5720, a PKA inhibitor, on ALP activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 12-well culture dishes and then cultured in growth medium (OS-) or osteogenic medium (OS+) supplemented with combinations of vehicle (M-), melatonin (M+) or KT 5720 (60 nM; PKI). Following a 10-day exposure, cells were assayed for ALP activity. As shown, no inhibition of melatonin-enhanced ALP activity was observed in the presence of KT 5720 (OS+M+/PKI) when compared to (OS+M+)-treated groups suggesting the lack of involvement of PKA in modulating melatonin's effects on ALP activity in hAMSCs. Each bar represents the mean \pm SEM of 4 experiments performed in duplicate. All data were analyzed by one-way ANOVA (F = 12.76; 5, 18 df; p < 0.0001) followed by a Newman-Keuls post hoc t-test where significance was defined as p < 0.05, where a = p < 0.05 when compared to OS-M-, b = p < 0.05 when compared to OS-M+; c = p < 0.05 when compared to OS+M-. Abbreviations: PKI = PKA inhibitor, KT5720.

Figure 11: The effect of GM 6001, a metalloproteinase inhibitor, on ALP activity in hAMSCs.

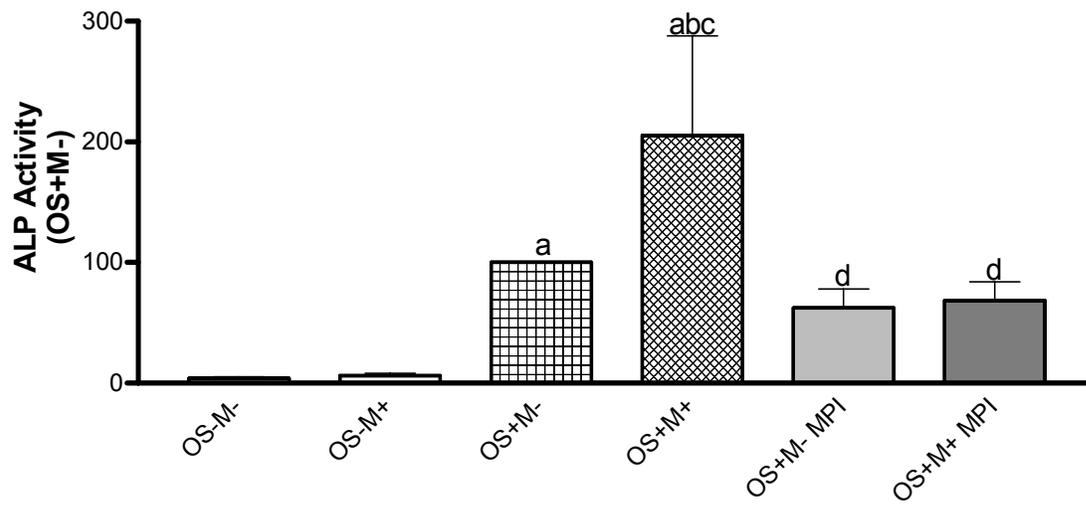


Figure 11: The effect of GM 6001, a metalloproteinase inhibitor, on ALP activity in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 12-well culture dishes and then cultured in growth medium (OS-) or osteogenic medium (OS+) supplemented with combinations of vehicle (M-), melatonin (M+) or GM 6001 (27 nM; MPI). Following a 10-day exposure, cells were assayed for ALP activity. As shown, co-incubation of GM 6001 with melatonin blocked the enhancing effects of melatonin indicating the involvement of the metalloproteinase in modulating melatonin's effects on ALP activity in hAMSCs. Each bar represents the mean \pm SEM of 4 experiments performed in duplicate. All data were analyzed by one-way ANOVA ($F = 4.53; 5, 18 \text{ df}; p < 0.01$) followed by a Newman-Keuls post hoc t-test where significance was defined a $p < 0.05$, where a = $p < 0.05$ when compared to OS-M-, b = $p < 0.05$ when compared to OS-M+; c = $p < 0.05$ when compared to OS+M-; d = $p < 0.05$ when compared to OS+M+. Abbreviations: MPI = metalloproteinase inhibitor, GM 6001.

12. ERK-P/ERK translocation patterns in hAMSCs

The translocation patterns and activity of ERK-P, normalized against total ERK protein, was assessed in the cytosol and nuclear fractions of hAMSCs stimulated with melatonin during and/or after the 10 day differentiation period. The purity of the nuclear fraction was assessed through DAPI staining for nucleic acids in both the cytosol and nuclear fractions. As shown in figure 12, hAMSCs incubated with OS+M+ for 10 days and rechallenged with 50 nM melatonin for 10 min induced a significant enhancement in nuclear ERK-P/ERK activity compared to any other treatment group. A corresponding decrease in ERK-P/ERK protein activity was observed in the cytosolic fraction of OS+M+/M+ treated cells. Additionally, hAMSCs incubated with OS+M+ and rechallenged with vehicle (OS+M+/M-) showed the highest amount of ERK-P/ERK protein activity in the cytosol (Fig 12B). These results indicate that hAMSCs incubated with OS+M+ during the 10 day differentiation period remain sensitive to melatonin rechallenge (OS+M+/M+) as assessed by nuclear translocation of activated ERK. Conversely, hAMSCs incubated with OS+M- during the 10 day differentiation period and rechallenged with 50 nM melatonin (OS+M-/M+) did not induce activated ERK translocation into the nucleus compared to hAMSCs incubated with OS+M- and rechallenged with vehicle (OS+M-/M-), indicating hAMSCs require melatonin to be present during the differentiation period to induce activated ERK translocation into the nucleus.

Figure 12: ERK-P/ERK translocation patterns in hAMSCs.

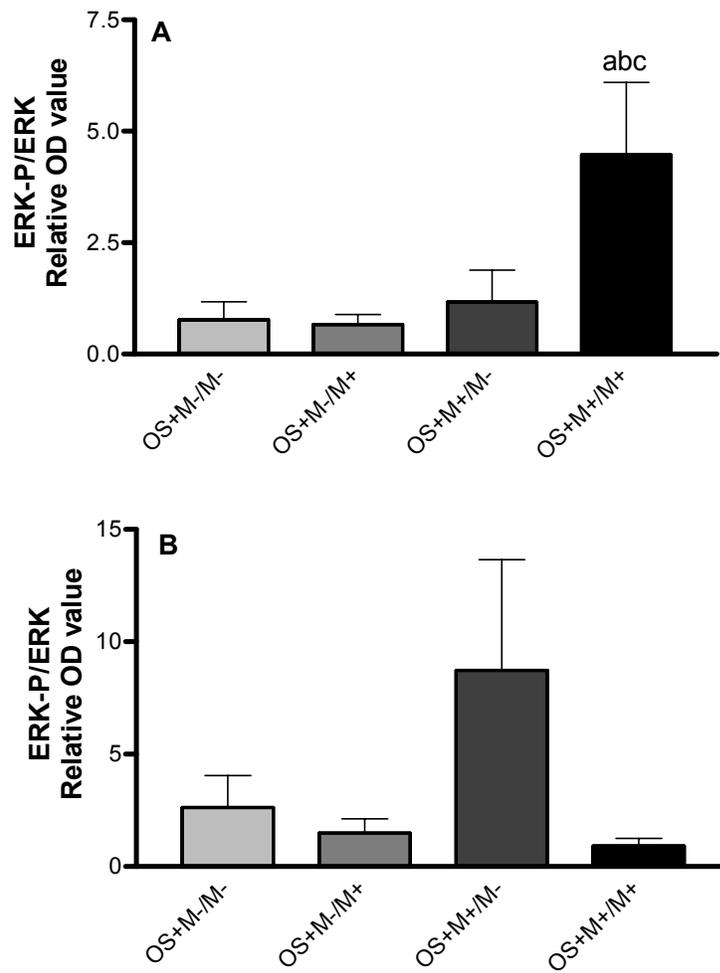


Figure 12: ERK-P/ERK translocation patterns in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 10 cm culture dishes and then grown in osteogenic medium with (OS+M+) or without (OS+M-) 50 nM melatonin for 10 days. Following the 10- day exposure, cells were rechallenged with either vehicle (/M-) or 50 nM melatonin (/M+) for 10 min and the nuclear (A) and cytosolic (B) fractions were separated as described. Equal amounts of protein were loaded onto a 4-20% gradient acrylamide gel and subjected to western blot analysis. Total ERK and ERK-P levels were detected using anti-ERK (1:10,000) or anti-ERK-P (1:1,000) antibodies. Proteins were visualized using ECL and the bands were quantified by densitometry using NIH Image software. Each bar represents the mean \pm SEM of 5-6 experiments. All data were analyzed by one-way ANOVA ($F = 4.07$; 3, 18 df; $p < 0.05$) followed by a Newman-Keuls post hoc t-test where significance was defined as $p < 0.05$.

13. Western blot analysis of proteins coupled to the immunoprecipitated MT₂ receptor expressed in hAMSCs

Immunoprecipitations of the MT₁ and MT₂ melatonin receptors and subsequent western blot analysis were performed to characterize proteins coupled to each receptor to reveal the signaling proteins involved in this melatonin-induced osteoblast differentiation process. Immunoprecipitations of the MT₁ melatonin receptor expressed in hAMSCs followed by western blot revealed no consistent coupling to the EGFR, Gi_{α2}, MEK, ERK or β-arrestins 1 and 2. This result corresponds with the finding that MT₂ melatonin receptors are driving these differentiation effects induced by melatonin. This is also supported in our previous findings showing that the MT₂ selective antagonist 4P-PDOT inhibits melatonin's ability to enhance ALP activity. Supporting this is the immunoprecipitation data shown in figure 13. hAMSCs incubated with OS+M- for 10 days and rechallenged with melatonin (OS+M-/M+) induced a significant increase in Gi_{α2}, β-arrestin-1, β-arrestin-2, and MEK coupling to MT₂ melatonin receptors relative to hAMSCs rechallenged with melatonin + luzindole (OS+M-/M+L), or vehicle (OS+M-/M-). Melatonin rechallenge did not enhance EGFR or ERK coupling to the MT₂ receptor relative to vehicle or melatonin + luzindole rechallenge.

Figure 13: Western blot analysis of proteins coupled to the immunoprecipitated MT₂ receptor expressed in hAMSCs.

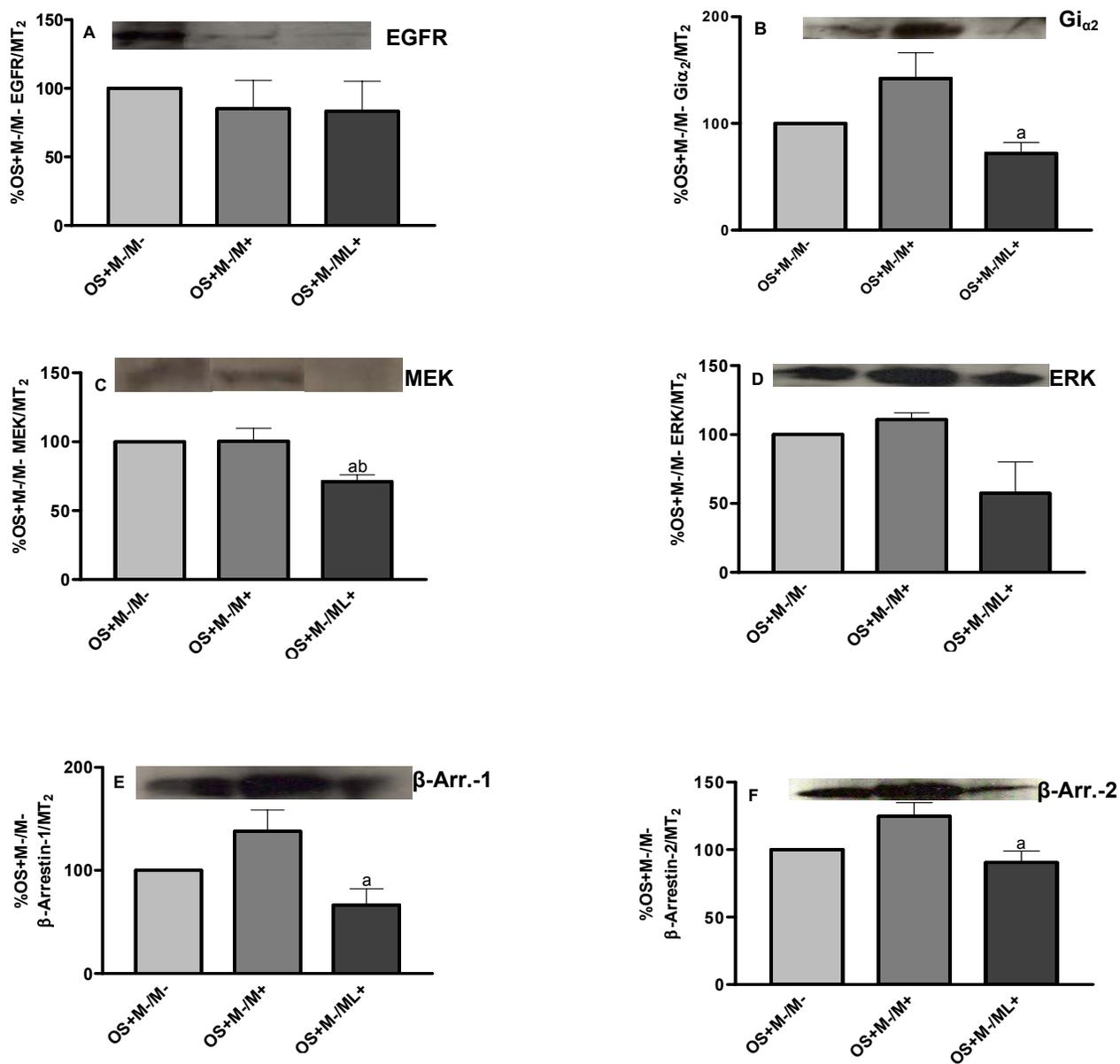


Figure 13: Western blot analysis of proteins coupled to the immunoprecipitated MT₂ receptor expressed in hAMSCs. Cells were seeded at a density of 3000 cells/cm² on 10 cm culture dishes and grown in osteogenic medium without melatonin (OS+M-) for 10 days. Following the 10- day exposure, cells were rechallenged with 0.1 μM melatonin (/M+), 0.1 μM vehicle (/M-), or 0.1 μM melatonin + 100.0 μM luzindole (/ML+) for 12 h and the MT₂ melatonin receptor was immunoprecipitated. Proteins coupled to the immunoprecipitated receptor were assessed via western blot for EGFR (A), Gi_{α2} (B), β-arrestin-1 (C), β-arrestin-2 (D), MEK (E), or ERK (F). Protein bands were quantified by densitometry using NIH Image software. The bands within an experiment were normalized against the OS+M-/M- controls. Each bar represents the mean ± SEM of 3 experiments. All data were analyzed by one-way ANOVA (B, F = 5.56; 2, 6 df; p < 0.05, C, F = 7.35; 2, 6 df; p < 0.05 E, F = 5.64; 2, 6 df; p < 0.05, F, F = 5.33; 2, 6 df; p < 0.05) followed by a Newman-Keuls post hoc t-test where significance was defined as p < 0.05 where a = p < 0.05 when compared to OS+M-/M+; b = p < 0.05 when compared to OS+M-/M-. Abbreviations: β-Arr.-1 = β-Arrestin-1, β-Arr.-2 = β-Arrestin-2.

B. Discussion

In this study we showed that melatonin, acting through melatonin transmembrane G-protein coupled receptors, enhances hAMSC differentiation into osteoblasts, as assessed by an increase in alkaline phosphatase enzymatic activity. The signaling mechanisms mediating melatonin's ability to enhance osteoblast differentiation was dependent on transactivation of EGF receptors and subsequent activation of the MAP-K signaling cascade. Immunoprecipitation of activated MT₂ receptors demonstrate that β -arrestin is coupled to the receptor, where it may play a role in receptor internalization and the formation of microdomains with the MAP-K signaling cascade.

Alkaline phosphatase (ALP) is an essential enzyme produced by osteoblasts involved in bone mineralization that can be monitored as an osteoblast-associated biomarker to assess osteoblast differentiation [84]. As hAMSCs differentiate into mature osteoblasts, ALP production increases [85-87]. Our data supports the findings of others' [43, 44], that an osteogenic medium (i.e., ascorbate, dexamethasone or β -glycerophosphate) and serum (i.e., growth factors), were essential for melatonin's enhancing effects on ALP activity. In the present study, melatonin did not independently enhance ALP activity with cells cultured in growth medium (OS-), but did enhance ALP activity with cells cultured in osteogenic medium (OS+).

Dexamethasone and ascorbate have been reported to independently enhance ALP activity in several immature bone progenitor models [110-114]. Perhaps, melatonin acting through the melatonin receptor and the mitogenic

signaling cascade, requires the induction of gene expression mediated through the synthetic glucocorticoid dexamethasone to mediate its osteoblast differentiation effects. Dexamethasone has been shown to independently induce osteoblast differentiation [113, 114] by acting through the cytosolic glucocorticoid receptor α (GR α). Once activated by dexamethasone, the GR α binds to nuclear GR binding elements (GREs) to induce gene expression of *Runx2*, *collagen1a1*, osteocalcin and ALP necessary for osteoblast differentiation [115, 116]. These enhancing effects of melatonin on ALP activity may occur through the mitogenic cascade that runs parallel to the GR α signaling pathway and that either (1) converges on the same DNA response elements targeted by GR α , or (2) activates genes to produce co-activators that then synergize with the regulatory elements produced in response to glucocorticoid exposure. Messenger RNA transcript levels of these genes implicated in osteoblast differentiation should be evaluated to further investigate the additive effect between melatonin and OS+ medium.

Ascorbic acid has additionally been shown to independently enhance ALP activity in periodontal ligament cells by enhancing the expression of $\alpha_2\beta_1$ integrin receptor and subsequent type I collagen extracellular matrix production [112]. Melatonin's inability to enhance ALP activity alone has been previously reported. Nakade and colleagues [44], find that serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with melatonin and 0.01% bovine serum albumin, does not significantly enhance ALP production in human bone cells. However, Roth and colleagues [43] report that melatonin concentrations as low

as 10 nM are able to stimulate ALP gene expression in preosteoblast and rat osteoblast-like osteosarcoma cells cultured in growth medium containing FBS, β -glycerophosphate and ascorbic acid.

Even though the identity of the melatonin receptor involved in inducing osteoblast differentiation in these other models was not determined, in the present study, the melatonin-enhancing effects on ALP activity in hAMSCs is strongly suspected to have occurred via MT₂ melatonin receptors. The involvement of melatonin receptors and G_i proteins in bone-related diseases has been documented [49]. Melatonin's effects on ALP activity seem to occur through an enhancement in osteoblast differentiation from pre-existing hAMSCs rather than through an increase in osteoblast proliferation, as OS+M+ treatment significantly reduces proliferation compared to negative control.

Typically, a slowing of cell proliferation precedes cellular differentiation [117, 118], however, the signaling mechanisms underlying these events are not clear. In many studies, MEK and ERK are implicated in cell proliferation [64, 119]. In murine MC3T3-E1 osteoblast-like cells, inhibition of MEK using U0126, blocks cellular proliferation but not the expression of osteoblastic markers ALP and osteocalcin. However, p38 inhibition by SB203580 blocks the expression of osteoblastic markers induced by serum without affecting cellular proliferation. The role of JNK in mediating these processes is not clear, but may regulate other markers of differentiation including collagen and osteocalcin [64]. Glucocorticoid receptors may also be involved. In mouse MBA-15.4 and human MG-63 preosteoblastic cell lines, chronic exposure to dexamethasone inhibits cell

proliferation and MEK/ERK activity via an up-regulation of MKP-1, a MAP-K phosphatase [119].

Melatonin's effects on cell proliferation are variable. Melatonin stimulates proliferation in human HOB-M bone cells and SV-HFO human osteoblastic cells [45]. In other studies, melatonin produces inhibitory responses in proliferation [100, 120]. Biphasic responses to melatonin on cell proliferation have been observed [104, 54, 120, 121] that are dependent upon melatonin concentration and the cell type examined. In our study, we observed a subtle, yet significant inhibition of cell proliferation in treatment groups containing melatonin, osteogenic medium, or both. The inhibition of proliferation in the (OS+M-) group could be mediated through dexamethasone's and/or melatonin's inhibitory effects on hAMSC proliferation. We do not believe this (OS+)-induced inhibitory effect on proliferation is mediated through the MEK/ERK pathway because hAMSCs incubated in osteogenic medium alone (OS+M-) did not show a decrease in MEK/ERK activity when compared to cells incubated in growth medium (OS-). The only decrease in MEK/ERK activity occurred when cells were cultured in osteogenic medium supplemented with melatonin (OS+M+). These data suggest that melatonin modulated this pathway and not factors within the osteogenic medium. Surprisingly, melatonin, when added in combination with osteogenic medium (OS+M+) did not further inhibit cellular proliferation when compared to (OS+M-)-treated groups. Perhaps, this level of inhibition was maximal and no further decrease in proliferation could occur.

The mechanisms underlying osteoblast differentiation are not clear,

however, past studies point to a role for the mitogen activated signaling cascade in modulating these processes. A number of recent investigations have implicated the p38 mitogen-activated protein kinase [63 - 65, 122] in regulating osteoblast differentiation. Suzuki and colleagues [63] reveal that the p38 specific inhibitor, SB203580, almost completely abolishes both ALP production and ALP mRNA expression in MC3T3-E1 osteoblast-like cells. This same group [64] show that factors within serum contribute to osteoblastic differentiation, in particular, factors that stimulate the MAP-Ks, ERK, p38 and JNK. Similarly, but in other studies, p38 inhibition by SB203580 completely abolishes bone morphogenetic protein (BMP)-induced osteocalcin gene expression and matrix mineralization in human trabecular bone-derived osteoblasts [122] and osteoblast markers in C3H10T1/2 and C2C12 mesenchymal cell lines [65]. Though not tested, we do not believe that p38 is involved in melatonin-induced hAMSC differentiation for two reasons: (1) in past studies, inhibition of p38 MAP-K does not block ascorbic acid-induced mesenchymal cell differentiation [63], (2) ERK-1/2 MAP-K inhibition, but not p38 MAP-K inhibition completely abolishes activation of the Runx2 transcription factor involved in controlling osteoblast differentiation [123], and (3) an inhibition of ALP activity in the presence of a MEK 1/2 inhibitor, PD98059, was observed in our system. Because ascorbate was one of the components in our osteogenic medium and ALP activity induced by melatonin was completely inhibited by PD98059, the mechanism underlying melatonin-induced hAMSC differentiation in the presence of osteogenic medium is probably through MEK and ERK and not through p38 MAP-K. The role of

ascorbic acid in inducing ALP activity may be through collagen and the $\alpha_2\beta_1$ integrin receptor [63].

Besides the present study, others show that the extracellular signal-related kinase (ERK) [65, 66, 124, 125] and G_i proteins [107, 125] are involved in osteoblast differentiation. For example, in one study using bone morphogenic protein (BMP) to induce osteoblast differentiation of a mesenchymal progenitor cell line (3H10T1/2), it is shown that BMP-2 induces the expression of ERK 1 and 2, which is blocked in a cell line expressing a dominant negative form of ERK 2 [66]. In another study using transfected cell models, it is shown that the addition of pertussis toxin blocks isoproterenol-induced or UK14303-induced activation of ERK mediated through α_2 ARs or β_{2A} ARs, respectively [107]. Likewise, in human fetal CRL-11372 preosteoblastic cells, ultrasound-induced activation of ERK and the osteoblastic markers, Cbfa1/Runx2 and osteocalcin, are shown to be pertussis toxin sensitive [125].

In our study, acute melatonin exposure increased ERK activity while reduced activity levels of both MEK and ERK 1/2 were observed when hAMSCs were exposed chronically to melatonin. Fully desensitized melatonin receptors may explain these dual effects in ERK activity. The fact that MEK inhibition also blocked the melatonin-induced increases in ALP activity back to (OS+M-)-treated levels, suggested MEK activation at the earlier stages of osteoblastic differentiation was essential for these melatonin-enhancing effects. As normal functioning melatonin receptors couple to an inhibition of adenylyl cyclase via G_i proteins and activate ERK, then a lack of a decrease in cAMP and PKA activity,

due to desensitization of this pathway, should inhibit MEK/ERK signaling. In some models of cellular differentiation, cAMP does play a role [68], while in others, it does not [65, 71]. Most notably, in murine osteoblasts, PGE₂, cholera toxin, or forskolin-mediated adenylyl cyclase activation fails to significantly enhance ERK 1/2 phosphorylation despite robust increases in intracellular cAMP [71]. Cyclic AMP-dependent mechanisms were similarly not involved in the melatonin enhancement of ALP activity in hAMSCs differentiated into osteoblasts because an inhibition of PKA by KT5720 did not modulate melatonin's enhancing effects.

The role that EGFRs and clathrin-mediated endocytosis play in modulating hAMSC differentiation into an osteoblast may be via their effects on MEK and ERK. In our study, acute melatonin exposure increased EGFR activity whereas an inhibition of EGFR activation during the differentiation process blocked any of melatonin's enhancing effects on ALP activity. Also, the metalloproteinase inhibitor, GM 6001, blocked melatonin's enhancing effects probably by preventing HB-EGF shedding and EGFR activation. In a previous study using transfected cell models, it is shown that EGFR transactivation by two different GPCRs, the α_{2A} AR and the β_2 AR, occurs. In addition, it is shown that both transactivation and internalization of EGFRs needs to occur in order for ERK 1/2 to be activated by either one of these receptors [107]. Similarly, but in PC12 cells, Piiper and colleagues [68] demonstrate that cAMP or forskolin-induced ERK 1/2 phosphorylation is blocked in the presence of an EGFR tyrosine kinase inhibitor, PD168393 implicating the importance of EGFR activation in modulating

MEK and ERK activity in these cells. Perhaps most compelling, hAMSC exposure to EGF but not PDGF, enhances both alkaline phosphatase activity and *in vitro* mineralization following 2-4 d and 9-12 d respectful exposures [67]. Similar to Piiper's findings, co-exposure of EGF with the MAP-K inhibitor U0126 completely inhibits the phosphorylation of ERK1 and ERK2, and completely abolishes the hAMSCs ability to differentiate into the osteoblast.

The mechanism(s) underlying EGFR transactivation by GPCRs may involve G-proteins [126, 127]. In COS-7 cells, activation of either endogenously-expressed LPA receptors by lysophosphatidic acid, or transiently transfected α_{2A} ARs by UK14304, induces the activation of EGFRs [126]. In this same study, it is shown that $G_{\beta\gamma}$ induces this phosphorylation of EGFRs through the adapter proteins, Shc and Grb2 [126]. Once activated, these adapter proteins interact with the intracellular SH2 domain of the EGFR to induce the recruitment of the guanine nucleotide releasing factor Sos to the plasma transmembrane. Once translocated to the membrane, Sos comes in close proximity to the small G-protein Ras, where it stimulates the exchange of GTP for GDP [128]. Once activated, Ras interacts with several effector proteins including Raf, inducing the activation of the MAP-K signaling cascade. More recently, and in an elegant set of experiments, it is shown that PTH-induced activation of ERK in cultured murine osteoblasts requires EGFR transactivation by promoting the release of EGF-like peptides through metalloproteinases [127]. Also, EGF and EGFRs have been shown to play an important role in bone development in transgenic mice [129-131]. Taken together, these studies show that GPCRs can modulate

MEK and ERK1/2 activity in some cells through pertussis toxin sensitive G-proteins and requires both EGFR activation and internalization.

Our study revealed that melatonin's enhancing effects on ALP activity in differentiating hAMSCs into osteoblasts is dependent upon clathrin-mediated endocytosis, MT₂ melatonin receptors, EGFRs, pertussis toxin-sensitive G-proteins, MEK 1/2 and, perhaps, metalloproteinases. How all of these proteins and events can be involved in this differentiation process can be best explained by an intriguing hypothesis put forth in a review [132] and as shown in Fig. 13. In this review, it is proposed that MEK and ERK (1/2) activity can be modulated by GPCRs via β -arrestin scaffolds and transactivation of EGFRs by metalloproteinases forming a scaffolding chain. The clathrin-mediated endocytosis is thought to occur to bring together these signaling components forming microdomains, which ultimately result in MEK/ERK 1/2 activation. This is thought to occur following the activation and phosphorylation of GPCRs by agonist and β -arrestin binding as well as G _{$\beta\gamma$} -mediated activation of metalloproteinases resulting in HB-EGF shedding and activation of EGFR. Perhaps, in differentiating hAMSCs exposed chronically to melatonin, scaffolds between phosphorylated and desensitized MT₂ melatonin receptors and β -arrestin are formed bringing together a signaling chain that results in the activation of ERK. Activated ERK can then translocate to various parts of the cell and modulate the activity of, as yet unidentified, proteins involved in cellular differentiation (Fig. 13). Additionally, the melatonin enhancing effects on ALP activity occurred when melatonin receptors were fully desensitized, implying that

MT₂ melatonin receptor desensitization is a necessary step in hAMSC differentiation into an osteoblast. A further characterization into the mechanisms underlying melatonin receptor desensitization will form the basis of future experiments. In addition, the effects of melatonin on hAMSC differentiation at the different stages of osteoblast development will also be examined.

Findings from the MT₂ immunoprecipitation studies support the involvement of G-proteins, β -arrestin, and MEK proteins in the melatonin receptor microdomain complex following agonist exposure. As expected, activation of the MT₂ receptor by melatonin resulted in an enhanced coupling to Gi_{α2} proteins.

Figure 14: Schematic representing melatonin-induced enhancement of ALP activity.

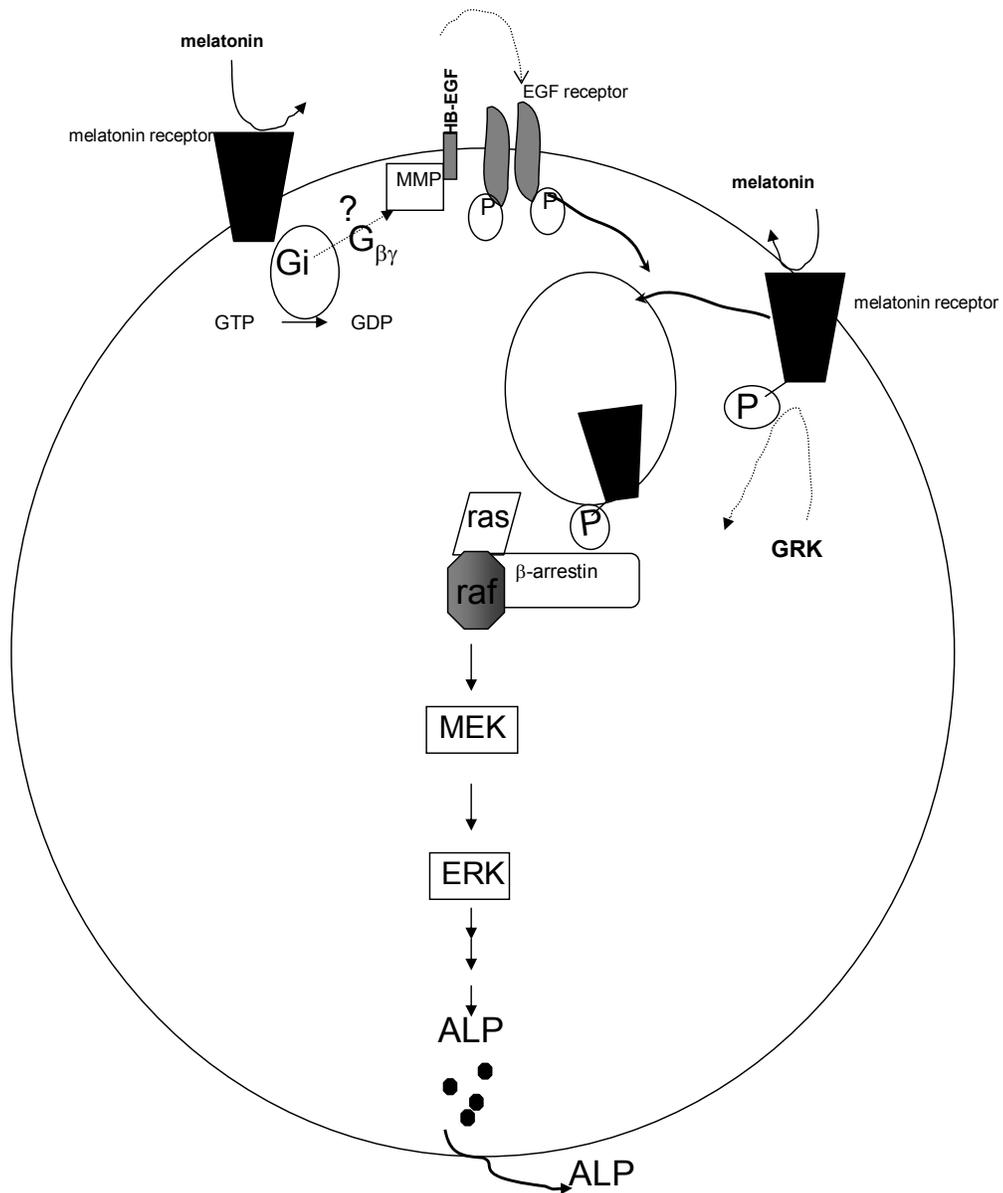


Figure 14: Schematic representing melatonin-induced enhancement of ALP activity. Prolonged melatonin exposure induces homologous GRK-mediated phosphorylation of the MT₂ receptor, with subsequent β -arrestin binding, and clathrin-dependent receptor internalization. Once internalized, β -arrestin scaffolds to the MAP-K pathway. Additionally, G _{$\beta\gamma$} -mediated activation of matrix metalloproteinases activates the EGF receptor and MAP-K pathway.

These results, coupled with the observation that pertussis toxin co-exposure abolished melatonin's ability to enhance ALP activity, strongly implicate the G proteins involvement in osteoblast differentiation. Future research should evaluate $G_{\beta\gamma}$ involvement in the osteoblast differentiation process, through the use of $G_{\beta\gamma}$ -specific inhibitors (GRK2-ct), or western blot assessment of the $G_{\beta\gamma}$ protein coupled to the immunoprecipitated MT_2 receptor.

Agonist-dependent increases in β -Arrestin-1 and β -arrestin-2 binding to the immunoprecipitated MT_2 melatonin receptor indicate the proteins' ability to form microdomains with the receptor. While both β -arrestin isoforms associate with the MT_2 melatonin receptor in an agonist-dependent manner, each protein may not have equal ability to form microdomains with the MAP-K signaling cascade. Previous research from Luttrell and colleagues [133] similarly indicate that β -arrestin-2 scaffolds bind to stimulated angiotensin II 1a receptors (AT1aR) and facilitates the assembly of the cRaf-1, MEK-1, and ERK2 microdomain. Our results show an agonist-mediated enhancement in MEK, but not ERK coupling to the immunoprecipitated MT_2 receptor. Perhaps in hAMSCs, upstream MEK proteins couple with the β -arrestin- MT_2 receptor microdomains to facilitate further downstream, uncoupled ERK activation. Despite the lack of agonist-dependent coupling to the microdomain complex, ERK phosphorylation is significantly enhanced in hAMSCs acutely treated with melatonin, indicating a modulation of ERK activity by the upstream microdomains. Additionally, considering that MDC prevented melatonin's ability to enhance ALP activity suggests that clathrin-mediated internalization processes are essential in effecting these processes.

The immunoprecipitation results also provide further support for the requirement of internalized receptors and the β -arrestin-mediated scaffolding domains to activate the MAP-K signaling pathway. These findings may explain the observation that desensitized melatonin receptors significantly enhanced ALP activity during the differentiation process. Following chronic melatonin stimulus, β -arrestin coupling to the receptor is enhanced, whereupon the $G_{i\alpha}$ and MEK proteins necessary to induce osteoblast differentiation are assembled into a scaffolding microdomain that facilitates enhanced signaling efficiency. β -arrestin siRNA constructs should be utilized to further evaluate whether these assembled microdomain complexes enhance osteoblast differentiation in the hAMSC.

Further evidence supporting increased signaling efficiency in β -arrestin-bound melatonin receptors was observed during assessment of ERK translocation. Interestingly, hAMSC exposed to OS+M+ treatment during the 10 day differentiation period and re-challenged with melatonin (OS+M+/M+) induced a significant increase in ERK-P activity within the nucleus, with a concomitant decrease in ERK-P activity in the cytosol. Similar ERK-P nuclear translocation patterns were not observed in hAMSCs differentiated in osteogenic medium containing vehicle followed by a melatonin rechallenge (OS+M-/M+). These translocation results support the notion that internalized melatonin receptors, coupled with β -arrestin scaffolding microdomains, facilitate melatonin intracellular responses. Perhaps phosphorylated ERK nuclear translocation indicates transient β -arrestin binding to the MT_2 receptor, as stable β -arrestin binding with the activated receptor is associated with cytosolic retention of activated ERK

levels [79]. In transfected COS-7 cells, it is shown that increased cytosolic translocation of ERK-P induces a more robust mitogenic response, proposedly by phosphorylating cytosolic kinases such as p90 RSK involved in transcriptional regulation. Accordingly, the melatonin-mediated reduction in cytosolic ERK-P may explain the observed attenuation in hAMSC proliferation. β -Arrestin's ability to enhance the nuclear translocation of ERK-P protein has been reported [74]. Isoproterenol treated COS-7 cells, transfected with the β_2 -adrenergic receptors, causes a significantly higher amount of nuclear ERK-P present in cells expressing β -arrestin-2. The physiological consequence of increased ERK-P levels in the nucleus is uncertain in hAMSCs, but may serve to activate Runx2, osterix and/or Egr-1; nuclear transcriptional factors that modulate hAMSC differentiation into the osteoblast [123, 134-137]. Considering that activation of these nuclear transcription factors has been shown to be essential for the differentiation of a fully functional osteoblast, then the enhanced ERK-P translocation may ultimately mediate melatonin's ability to enhance osteoblast differentiation from a hAMSC.

Despite the observations that EGFR activation is necessary for melatonin to enhance ALP activity and acute melatonin exposure significantly increases EGFR phosphorylation, melatonin exposure did not enhance EGFR coupling to the immunoprecipitated MT_2 receptor. These data indicate that EGFRs are not physically associated with the β -arrestin scaffolding microdomain. This finding is unique considering past studies have shown agonist stimulation of the β_2AR causes the formation of a complex containing β_2AR , EGFR, β -arrestin-1, and the

non-receptor tyrosine kinase c-Src [132]. Transactivation of the EGFR by melatonin may therefore progress independently of the β -arrestin scaffold in differentiating hAMSCs through $G_{\beta\gamma}$ -mediated activation of transmembrane matrix metalloproteinases. Two separate results support this theory. Co-exposure of hAMSCs with either pertussis toxin or the broad-spectrum matrix metalloproteinase inhibitor inhibited melatonin-mediated enhancement in ALP activity. By catalyzing the ADP-ribosylation of the G_i heterotrimeric proteins, pertussis toxin prevents the G protein heterotrimer from interacting with the receptor. Thus, the $G_{\beta\gamma}$ subunit is inhibited from interacting with the matrix metalloproteinase. Secondly, inhibition of the matrix metalloproteinase prevents melatonin's ability to enhance ALP activity, perhaps by preventing the liberation of the HG-EGF agonist responsible for EGFR activation. Collectively, these findings indicate that the stimulated EGFR may activate the MAP-K protein by assembling β -arrestin microdomains, perhaps through the recruitment of adaptor proteins, Shc and Sos, and small G-proteins like Ras.

Overall, the results from this study reveal that melatonin plays a significant role in human osteoblast development. It sheds some novel insight as to the role of MT_2 melatonin receptors and the MEK/ERK (1/2) signaling cascade on mediating this process. Perhaps, therapies targeted at melatonin receptors may prove to be effective in preventing osteoporosis.

IV. CONCLUSIONS

Findings from this study support the notion that melatonin enhances bone formation through the enhancement of osteoblast differentiation in a clinically relevant human adult mesenchymal stem cell line. While melatonin may also enhance bone formation by impeding bone-resorbing osteoclast activity, our findings refute claims that melatonin stimulates bone formation independent of osteoblasts. These findings indicate that the decrease in circulating melatonin levels with age makes humans more susceptible to osteoporosis formation. The use of melatonin replacement therapy may therefore prove an effective manner to retard osteoporosis formation with age.

Our results also reinforce the prominent role the mitogen activated protein kinase cascade plays in differentiation pathways. In addition to modulating differentiation, the MAP-K pathway has also been reported to regulate proliferation. The observation that prolonged melatonin exposure reduces MAP-K activation indicates a homeostasis mechanism to regulate the variety of essential cellular functions MAP-K modulates. Additionally, the identification that melatonin-mediated enhancement in osteoblast differentiation is dependent upon epidermal growth factor receptors but not protein kinase A activation identifies potential upstream targets of the MAP-K pathway that may have future drug design utility.

Finally, our results implicate β -arrestin scaffolding to connect components of the signaling cascade. This observation supports a growing body of evidence that intermediate agonist exposure, classically thought to induce a waning

agonist response, may briefly allow optimal conditions for protein scaffolding and subsequent signal transduction.

V. APPENDICIES

A. Buffers

Western Blot Assay Buffers:

Lysis buffer, pH = 7.5

20 mM Tris-HCl, adjust pH to 7.5
150 mM NaCl
1% Triton-X 100
1 mM EDTA
1 mM EGTA
1X protease inhibitors

Add dH₂O to 1000 ml, adjust pH to 7.5
Store at -20°C until further use

2 X SDS loading buffer

0.2% bromophenol blue
200 mM DTT
20% glycerol
4% SDS
100 mM Tris Cl

Add dH₂O to 500 ml, 2 x SDS loading buffer lacking DTT can be stored at 4°C. DTT should be added before buffer is to be used.

Tris-glycine electrophoresis buffer, pH = 8.3

25 mM Tris
250 mM glycine
0.1% SDS

Add dH₂O to 1000 ml, adjust pH to 8.3
Store at 4°C

Tris-buffered Saline

25 mM Tris
140 mM NaCl
2.7 mM KCl

.Add dH₂O to 1000 ml, adjust pH to 7.4
Store at 4°C

Transfer Buffer

39 mM glycine
48 mM Tris base
0.037% SDS
20% methanol

Add dH₂O to 1000 ml
Store at 4°C

Nuclear/Cytosol Fractionation:

Lift Buffer

50 mM β glycerophosphate
1 mM DTT
1 mM EDTA
1.5 mM EGTA
1 mM Na₃VO₄
1 X complete mini protease inhibitors

Add dH₂O to 100 ml
Store at 4°C

Lysis buffer

40 mM β glycerophosphate
1 mM DTT
10 mM EGTA
20 mM HEPES
2 mM Na₃VO₄
1.0% Nonidet P-40

Add dH₂O to 100 ml
Store at 4°C

Nuclear Extraction Buffer

50 mM β glycerophosphate

1 mM DTT
200 μ M EDTA
25% glycerol
1.5 mM MgCl_2
420 mM NaCl
500 μ M Na_3VO_4

Add dH_2O to 500 ml
Store at 4°C

Immunoprecipitation Buffer:

Lift buffer, 7.4

10 mM KPO_4
1 mM EDTA

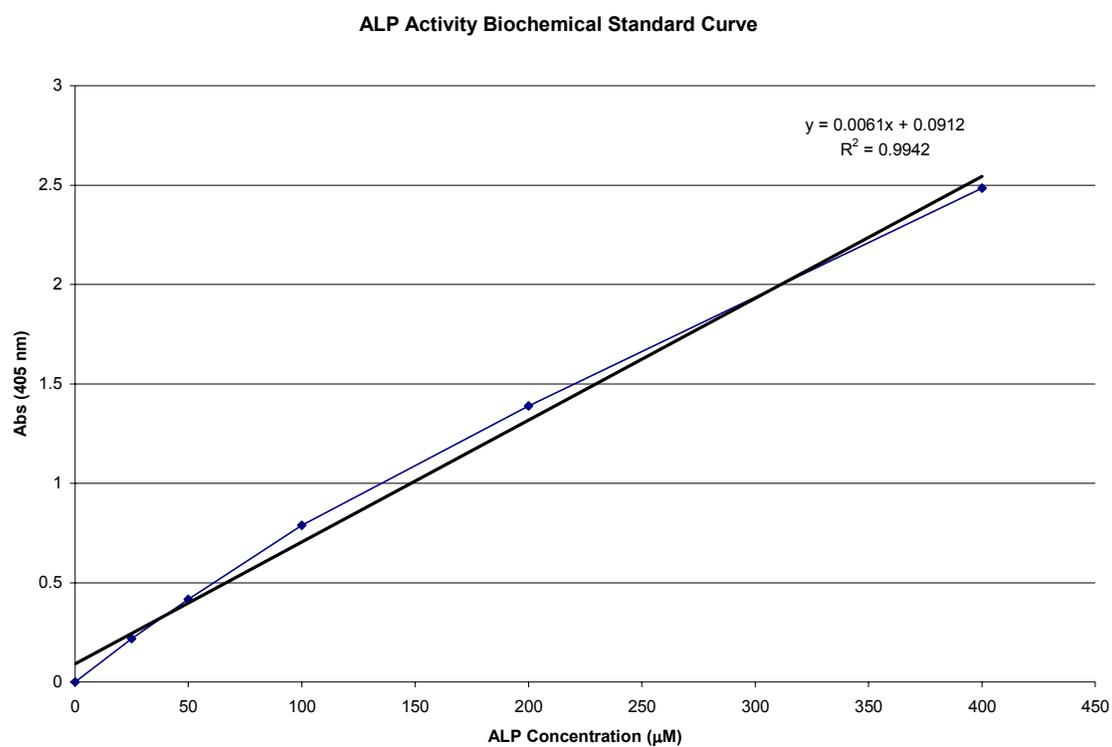
Add dH_2O to 500 ml, adjust pH to 7.4
Store at 4°C

Buffer A

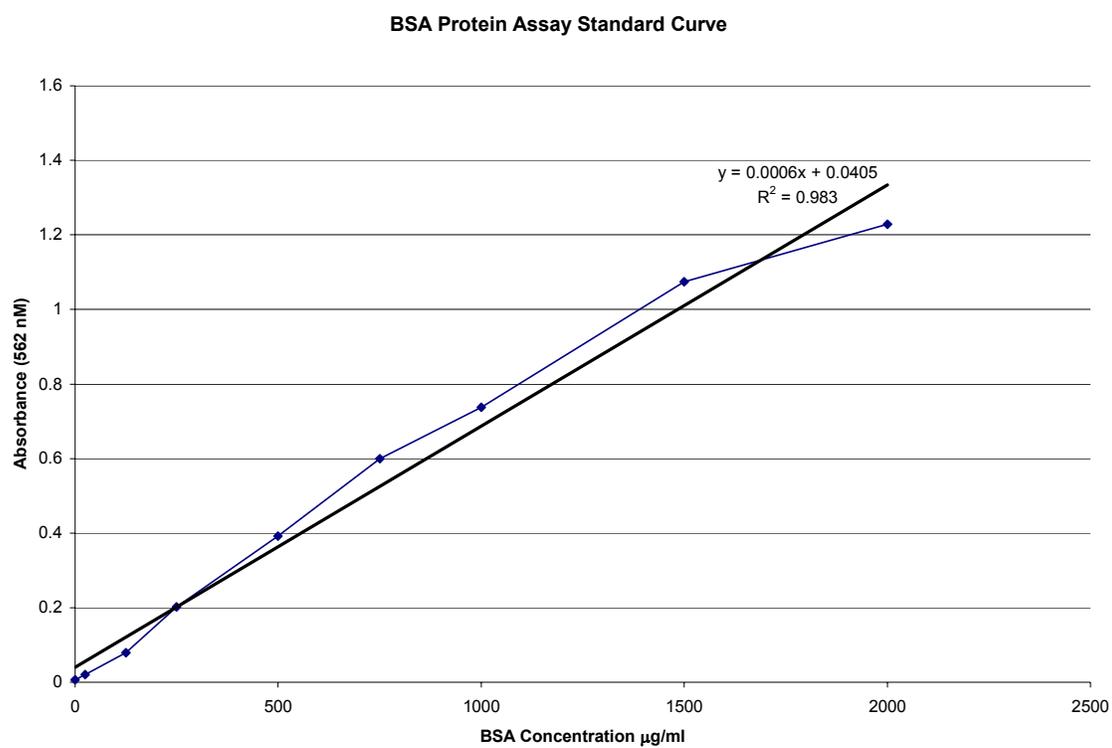
0.5 M EDTA
12 mM MgCl_2
1 M Tris containing
1 X complete mini protease inhibitors

Add dH_2O to 500 ml
Store at 4°C

B. Alkaline Phosphatase Activity Standard Curve



C. Bovine Serum Albumin Protein Standard Curve



D. Live/Dead Cytotoxicity Analysis

Treatment	(Live/Dead)/OS+M-
OS+M-	100
OS+M- PTX	149
OS+M- LUZ	100
OS+M- 4P-PDOT	96
OS+M- MEK I	168
OS+M- EGFR I	100
OS+M- MDC	103

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VII. ABSTRACT

Current osteoporosis therapy is directed toward inhibiting the activity of bone-resorbing osteoclasts. Decreased bone producing osteoblast efficiency however, is largely responsible for osteoporosis development. Therefore, novel osteoporosis treatments should stimulate osteoblast formation and activity. Accordingly, the purpose of this study was to determine melatonin's role and subsequent mechanism(s) involved in differentiating human adult mesenchymal stem cells (hAMSCs) into osteoblasts as assessed by alkaline phosphatase activity (ALP) and determine whether receptor compartmentalization facilitates these signaling mechanisms. Melatonin significantly increased osteogenic medium-induced alkaline phosphatase activity, an enzyme associated with functional osteoblasts, indicating melatonin enhances osteoblast differentiation. The mitogen activated protein kinase signaling mechanisms has been implicated in osteoblast differentiation. Based on these findings, we hypothesized that melatonin acts through the mitogen activated protein kinase signaling cascade to enhance hAMSC differentiation into an osteoblast as assessed by alkaline phosphatase activity. Acute melatonin exposure to hAMSC stimulated extracellular regulated kinase (ERK) phosphorylation without affecting total ERK protein levels. Conversely, co-incubation with melatonin and the MEK inhibitor PD98059 prevented the melatonin-mediated enhancement in ALP activity in hAMSCs. Taken together, these findings indicate melatonin enhances ALP activity via activation of the mitogen activated protein kinase (MAP-K) cascade. Protein Kinase A (PKA), epidermal growth factor receptor (EGFR), and matrix

metalloproteinase (MMP) activation were evaluated as potential upstream inputs of the MAP-K activation. Subsequent co-incubation of melatonin with specific PKA, EGFR, and MMP inhibitors revealed that EGFR and possibly MMP but not PKA activate the MAP-K cascade in these cells. The potential for compartmentalization of the melatonin receptor and associative signaling pathways was assessed by immunoprecipitation analysis. The MT₂ receptor was immunoprecipitated in the presence of vehicle, melatonin or melatonin + luzindole and the pellet was subjected to western blot analysis using antibodies against G_i, EGFR, beta arrestin 1,2, MEK and ERK (1/2). Stem cells exposed to melatonin showed that MT₂ receptors do complex with G_{iα2}, beta arrestin, and MEK in an agonist-dependent manner. These findings indicate in differentiating hAMSCs exposed chronically to melatonin, beta-arrestin scaffolds form with MT₂ receptors bringing together a signaling chain that may facilitate activation of the MAP-K cascade and hence, their differentiation into osteoblasts.