Examination of Glucocorticoid Treatment on Bone Marrow Stroma: Implications for Bone Disease and Applied Bone Regeneration

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(ABSTRACT)

Long-term exposure to pharmacological doses of glucocorticoids has been associated with the development of osteopenia and avascular necrosis. Bone loss may be partially attributed to a steroid-induced decrease in the osteoblastic differentiation of multipotent progenitor cells found in the bone marrow. In order to determine if there is a change in the osteogenic potential of the bone marrow stroma following glucocorticoid treatment, Sprague-Dawley rats were administered methylprednisolone for up to six weeks, then sacrificed at 0, 2, 4, or 6 weeks during treatment or 4 weeks after cessation of treatment. Femurs were collected and analyzed for evidence of steroid-induced osteopenia and bone marrow adipogenesis. Although glucocorticoid treatment did inhibit bone growth, differences in ultimate shear stress and mineral content were not detected. The volume of marrow fat increased with increasing duration of treatment, but returned to near control levels after cessation of treatment. Marrow stromal cells were isolated from tibias, cultured in the presence of osteogenic supplements, and analyzed for their capacity to differentiate into osteoblast-like cells *in vitro*. Glucocorticoid treatment diminished the absolute number of isolated stromal cells, but did not inhibit the relative levels of bone-like mineral deposition or osteocalcin expression and secretion.

Although pharmacological glucocorticoid levels induce bone loss *in vivo*, physiologically equivalent concentrations have been shown to enhance the formation of bone-like tissue *in vitro*. However, glucocorticoids have also been reported to inhibit proliferation and type I collagen synthesis in marrow stromal cell cultures. In order to assess the effects of intermittent dexamethasone treatment on the progression of osteogenesis in rat marrow stromal cell culture, this synthetic glucocorticoid was removed from the culture medium after a variable period of initial supplementation. Cell layers were analyzed for total cell number, collagen synthesis, phenotypic marker expression, and matrix mineralization. Prolonged supplementation with

dexamethasone decreased proliferation, but did not significantly affect collagen synthesis. Furthermore, increased treatment duration was found to increase bone sialoprotein expression and mineral deposition. The duration of glucocorticoid treatment may be a key factor for controlling the extent of differentiation *in vitro*.

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Chapter 1:
Introduction

1.1 General background on glucocorticoids

Glucocorticoids, also known as corticosteroids, are one of five major classes of cholesterol-derived steroid hormones, which also include progestagens, mineralocorticoids, androgens, and estrogens. Naturally circulating glucocorticoids – such as cortisol (hydrocortisone) in humans and corticosterone in rats (Figure 1.1) – are synthesized within the mid-zone of the adrenal cortex, the zona fasciculata, in response to adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary gland [Baxter and Rousseau, Glucocorticoid Hormone Action, 1979]. ACTH secretion is induced by various physical and psychological stimuli, including hypoglycemia, fear, pain, heavy exercise, and infection. Glucocorticoids are key factors in the stress response within animals: they are known to alter carbohydrate metabolism, promote gluconeogenesis and glycogen synthesis, and enhance both fat and protein degradation *in vivo* [Stryer, Biochemistry, 1999]. These steroid hormones also possess anti-inflammatory and immunosuppressive properties. Consequently, they are administered at pharmacological doses (i.e., producing plasma concentrations equivalent to $>10^{-7}$ M cortisol) for the treatment of inflammatory disorders (e.g., rheumatoid arthritis, asthma) and to suppress the rejection response following organ transplantation [Canalis and Giustina, 2001].

The mechanisms of glucocorticoid action allow for these steroid hormones to influence the growth, differentiation, and metabolic activity of cells in multiple tissues. Glucocorticoids, like other cholesterol derivatives, are lipid-soluble and may diffuse through the plasma membrane without the aid of a protein channel. They regulate transcriptional activity by acting through a specific cytosolic receptor (Figure 1.2). This 777 amino acid protein – a member of the nuclear receptor superfamily – consists of a transactivation domain at the amino terminus, a DNA-binding domain with a C_4 zinc-finger motif, and a hormone-binding domain at the carboxyl terminus [Lodish et al., Molecular Cell Biology, 2000]. In the absence of glucocorticoid, the cytosolic glucocorticoid receptor (cGR) is retained in an inactive state – with high affinity for ligand – as part of a complex with molecular chaperones (e.g., heat shock protein Hsp90) [McLaughlin et al., 2002]. The cGR undergoes a conformational change upon binding with its ligand, and the resulting hormone-receptor complex translocates into the nucleus

[Picard and Yamamoto, 1987], where it can regulate the transcription of multiple genes in a variety of cell types. Transcriptional activation most likely occurs through direct binding of a cGR homodimer to a cognate DNA sequence (glucocortocoid response elements, or GREs) near the promoter site of the target gene [Beato, 1995]. Alternatively, cGRs can repress transcription either by interacting with negative glucocorticoid response elements (nGREs) [Sakai et al., 1988] – hindering the formation of transcription pre-initiation complexes at overlapping promoters – or by directly binding with other transcription factors and inhibiting their function [Beato, 1995; Akerblom et al., 1988]. Glucocorticoids may also affect protein translation by altering mRNA transcript stability [Diamond and Goodman, 1985; Petersen et al., 1989]. In addition to cytosolic receptors, recent studies have demonstrated the existence of membrane-bound glucocorticoid receptors (mGRs) [Chen et al., 1999a; Chen et al., 1999b; Gametchu et al., 1999]. By affecting secondary messenger systems (e.g., G-protein-coupled protein kinase pathways), glucocorticoids may act through mGRs to cause a more rapid cellular response (on the order of minutes) than is possible with genomic effects [Chen and Qiu, 1999].

1.2 Glucocorticoid action on skeletal function

1.2.1 *Bone disease secondary to glucocorticoid administration*

Over the past few decades, organ transplantation has developed into an effective therapy for end-stage renal, hepatic, cardiac, and pulmonary disease [Rodino and Shane, 1998]. The success rates for kidney, liver, heart, pancreas, lung, and bone marrow transplantation have improved substantially with the aid of immunosuppressive drug regimens. Without such regimens, transplant recipients typically mount a rejection response to their allograft(s), which is mediated by CD4 T cell recognition of foreign antigens from the allograft [Denton et al., 1999]. Current immunosuppresive therapies target T cell activation, cytokine production, and clonal expansion [Suthanthiran and Strom, 1994]. Glucocorticoids are included in most post-transplantation regimens [Rodino and Shane, 1998], inhibiting T cell activation as well as macrophage-mediated tissue injury by inhibiting cytokine production within both cell types [Denton et al., 1999]. A standard immunosuppressive procedure involves the use of initial and maintenance regimens to prevent allograft rejection, as well as short treatments with more aggressive regimens to combat episodes of acute rejection [Denton et al., 1999].

The lack of tissue specificity is a major drawback to current immunosuppressive therapies. Glucocorticoids are known to affect the cardiovascular, endocrine, gastrointestinal, ophthalmic, and musculoskeletal organ systems [Axelrod, 1976]. Glucococorticoid regimens have been reported to produce undesired side effects such as weight gain, hyperglycemia, and hypertension [Andersson and Goodkin, 1998]. Their effects on bone include osteoporosis and avascular necrosis. Of the two conditions, glucocorticoid-induced osteoporosis occurs at a much higher frequency and is marked by reduced bone mass, altered bone architecture, and an increased incidence of fracture from minimal trauma [Grotz et al., 2001]. The general loss of bone mineral density (BMD) is a condition known as osteopenia. For adult patients exposed to glucocorticoid excess for more than five years, approximately 30% develop fractures due to an osteopenic condition [Manelli and Giustina, 2000; Reid, 1997]. Regions of trabecular (cancellous) bone are affected to a larger extent than cortical bone [Adinoff and Hollister, 1983; Rickers et al., 1984]. Thus the incidence of fracture is substantially higher in these regions, particularly in the lumbar vertebrae and at the epiphyses (ends) of long bones [Braith et al., 2000; Canalis, 1996; Rodino and Shane, 1998].

The extent of osteopenia is directly related to both the dose and duration of glucocorticoid therapy [Van Staa et al., 2000]. Doses greater than 10 mg/day of prednisolone, or its equivalent, yield significant bone loss [Garton and Reid, 1993; Olbricht and Benker, 1993; Rodino and Shane, 1998]. Bone loss is most pronounced during the first 12 months of therapy, followed by a slow but continuous decline in BMD [Lo Cascio et al., 1990; Saito et al., 1995]. The overall effects appear to be independent of such factors as age, race, and gender [Dykman et al., 1985; Hahn and Mazzaferri, 1995]. Bone mass may be either totally or partially restored following cessation of treatment [Lufkin et al., 1988; Pocock et al., 1987; Rizzato et al., 1993]. Unfortunately, the period of rapid bone loss coincides with the period when immunosuppressive therapy is especially indispensable for preventing rejection.

Non-traumatic avascular necrosis of bone, also known as osteonecrosis, is another complication of glucocorticoid administration and is associated with high doses of the steroid hormones following transplantation [Bradbury et al., 1994; Miyanishi et al., 2002]. Avascular necrosis tends to occur at the epiphyses of long bones, particularly within the femoral head. Femoral head necrosis develops within two years of treatment for about 20% of patients given high doses of glucocorticoids [Eberhardt et al., 2001]. If left untreated, progressive necrosis may

lead to femoral head collapse. Total hip replacement is the primary therapeutic option after joint destruction [Mankin, 1992].

The pathological mechanisms of avascular necrosis are less understood than that of glucocorticoid-induced osteoporosis. Some investigators have proposed that an enlargement of marrow fat volume leads to increased intraosseous pressure [Miyanishi et al., 2002; Wang et al., 1977], which in turn compresses thin-walled blood vessels and restricts blood flow [Drescher et al., 2001]. The resulting ischemia causes necrosis within the affected region of bone. This mechanism is supported by reports of increased marrow fat volume [Vande Berg et al., 1999] and intraosseous pressure [Zizic et al., 1986] in patients with osteonecrosis. Alternatively, a glucocorticoid-induced increase in circulating lipids may lead to occlusion of small blood vessels, a process known as fat embolization [Fisher, 1978]. However, the evidence linking this mechanism to glucocorticoid excess is limited [Eberhardt et al., 2001].

1.2.2 *Direct effects of glucocorticoids on bone metabolism*

Glucocorticoids influence bone and mineral homeostasis through both direct and indirect actions. Direct actions refer to mechanisms by which glucocorticoids directly alter the metabolic activity of cells that synthesize and remodel bone. Under normal conditions, bone is continuously resorbed by multinucleated osteoclasts that advance along the targeted skeletal surface. Osteoblasts are recruited to synthesize new bone matrix (osteoid) at the resorption cavities [Buckwalter et al., 1996b]. Together, these coupled groups of osteoblasts and osteoclasts form temporary anatomic structures known as basic multicellular units (BMUs). Total bone mass is conserved during bone turnover through a balance between BMU resorption and formation. An uncoupling of these processes has been demonstrated with pharmacological levels of glucocorticoids, which reduce the rate of bone formation and possibly increasing the rate of bone resorption. Changes in bone turnover rate have been demonstrated by histomorphometric analysis after glucocorticoid treatment [Dempster, 1989; Reid, 1997; Weinstein et al., 2000]. Histological evidence indicates significant decreases in osteoid surface and mineral apposition rate [Dempster, 1989]. Whether bone resorption is actually enhanced remains controversial [Dempster et al., 1997; Diamond et al., 1997]. However, the inhibition of bone formation is thought to be central to the pathogenesis of glucocorticoid-induced osteoporosis [Canalis and Giustina, 2001]. Reduced serum levels of osteocalcin, a bone-specific

matrix protein, provide biochemical support for reduced bone formation [Chiodini et al., 1998; Pearce et al., 1998; Peretz et al., 1989].

The inhibition of new bone synthesis by glucocorticoids may be attributed to changes in the genesis, metabolic activity, and programmed cell death (apoptosis) of osteoblasts [Canalis, 1996]. First, pharmacological glucocorticoid levels inhibit the induction of progenitor cells into the osteoblastic lineage [Weinstein et al., 1998a]. The precursors for osteoblasts, adipocytes, chondrocytes, hematopoiesis-supportive stromal cells, and other connective tissue phenotypes are derived from putative mesenchymal stem cells [Beresford, 1989; Owen, 1988; Caplan, 1991]. These stem cells and their progeny have a complex hierarchy [Caplan, 1994]. Studies using marrow stroma-derived cell cultures suggest that osteoblasts and adipocytes share a common precursor [Bennett et al., 1991] and that the relationship between osteogenesis and adipogenesis is reciprocal within the marrow stroma [Beresford et al., 1992; Cui et al., 1997]. Therefore, glucocorticoids may reduce osteogenesis by directing progenitor cells to differentiate along the adipocytic lineage, inhibiting the recruitment of new osteoblasts for bone remodeling and repair. This mechanism may account for the increase in the volume of fatty marrow that has been associated with glucocorticoid-induced osteonecrosis in both humans [Vande Berg et al., 1999] and animals [Kwai et al., 1985; Wang et al., 1977].

Second, glucocorticoids suppress the proliferation and differentiated function of cells already committed to the osteoblastic lineage [Pereira et al., 2001]. These effects may be partially attributed to glucocorticoid regulation of the insulin-like growth factor (IGF) axis [Canalis and Giustina, 2001; Patschan et al., 2001]. The cytokines IGF-I and IGF-II are known to have anabolic effects on bone, stimulating proliferation of cells from the osteoblastic lineage [Hock et al., 1988] and inducing type I collagen synthesis by osteoblasts through autocrine signaling [Canalis, 1996]. Glucocorticoids inhibit the transcription of IGF-I within osteoblasts by the regulation of certain CCAAT-enhancer binding proteins, which associate with a response element on the IGF-I exon 1 promoter site [Delany et al., 2001]. Glucocorticoids also have been shown to repress the transcription of IGF-binding protein-5 (IGFBP-5) [Gabbitas et al., 1996], which is known to potentiate the effects of IGF-I [Andress and Birnbaum, 1992].

In addition to IGF-axis effects, glucocorticoids directly affect the synthesis of bone matrix proteins by osteoblasts. They repress the expression of type I collagen – the primary structural protein of bone matrix – by reducing its transcription and destabilizing α1(I)

procollagen mRNA [Delany et al., 1995a]. Glucocorticoids also stimulate collagenase 3 expression through the stabilization of its mRNA transcript [Delany et al., 1995b]. Collagenase 3, or matrix metalloproteinase 13, degrades type I collagen fibrils [Delany et al., 1995b; Zhao et al., 1999] and has been shown to degrade IGFBP-5 [Thrailkill et al., 1995].

Third, it is thought that glucocorticoid-enhanced apoptosis of both mature osteoblasts and osteocytes contributes to bone degeneration [Eberhardt et al., 2001; Weinstein et al., 1998a]. Under normal physiological conditions, a fraction of the osteoblasts from BMUs differentiate into flattened lining cells on the skeletal surface, while others become buried in the mineralized matrix during bone formation and terminally differentiate into osteocytes [Weinstein and Manolagas, 2000]. However, the majority (as many as 65%) are thought to die by apoptosis [Jilka et al., 1998]. Glucocorticoid administration has been reported to increase osteoblast apoptosis in murine trabecular bone, and increased apoptosis of both osteoblasts and osteocytes was observed in patients diagnosed with glucocorticoid-induced osteoporosis [Weinstein et al., 1998a]. Furthermore, glucocorticoid administration has been shown to increase osteocyte apoptosis in femoral head sections from patients diagnosed with avascular necrosis [Weinstein et al., 1998b]. Osteocytes remain connected to osteoblasts and lining cells by processes that extend through channels in the lamellar bone called canaliculi [Buckwalter et al., 1996a]. The network of osteocytes and canaliculi may have an important mechanosensory role in detecting microdamage and directing the remodeling process [Shapiro, 1988; Weinstein and Manolagas, 2000]. Therefore, increased osteocyte apoptosis may lead to an accumulation of microdamage and the development of atraumatic fractures.

In summary, the primary mechanism of glucocorticoid-induced osteoporosis is most likely an inhibition of bone formation, resulting from reductions in osteoblast number and metabolic activity (Table 1.1). The cummulative effects of reduced osteoblast recruitment, inhibited osteoblast proliferation, and increased osteoblast apoptosis by glucocorticoids are a decreased rate of new bone formation and a loss of healthy bone. For those osteoblasts still available to form bone, glucocorticoids inhibit IGF-I expression and type I collagen synthesis.

1.2.3 *Indirect effects of glucocorticoids on bone metabolism*

Glucocorticoids also have significant indirect effects on bone and mineral homeostasis. They alter systemic calcium balances, most likely through a combination of decreased intestinal

calcium absorption and increased renal calcium excretion [Canalis, 1996; Rodino and Shane, 1998]. Diminished calcium absorption at the intestine may be caused by a decrease in active transcellular transport, a decrease in vesicle uptake at the brush border (the densely packed microvilli on the apical surface of intestinal epithelial cells), or reduced synthesis of calcium binding proteins [Feher et al., 1979; Kimberg et al., 1971; Shultz et al., 1982]. The increased urinary elimination of calcium, hypercalciuria, may be the result of diminished tubular reabsorption [Reid and Ibbertson, 1987; Reid, 1997]. The resulting calcium deficiency may lead to increased resorption and loss of bone mass.

Through the decrease in systemic calcium levels, glucocorticoids may also promote secondary hyperthyroidism, elevating levels of parathyroid hormone (PTH) in the serum [Compston et al., 1996; Gennari et al., 1984; Lukert and Raisz, 1990]. However, contradictory studies have reported no increase in the serum levels of PTH after glucocorticoid administration [Hahn et al., 1979; Hattersley et al., 1994; Luengo et al., 1991; Paz-Pacheco et al., 1995; Slovik et al., 1980]. Instead, these steroids may alter the pattern of PTH release, inducing a more pulsatile secretion [Manelli et al., 2001]. PTH is known to regulate the release of calcium from the bone matrix and by stimulating osteoclastic differentiation (thus promoting bone resorption) [Buckwalter et al., 1996b]. Osteoblasts are thought to mediate the response of osteoclast precursor cells to PTH [McSheehy et al., 1986]. Recent studies have shown that PTH stimulates synthesis of receptor activator of nuclear factor-κβ ligand (RANK-L), a transmembrane protein expressed on osteoblastic lineage cells [Lacey et al., 1998; Lee and Lorenzo, 1999]. RANK-L – also known as osteoclast differentation factor (ODF), osteoprotegerin ligand (OPGL), and tumor necrosis factor-related activation-induced cytokine (TRANCE) – is a member of the tumor necrosis factor (TNF) family [Suda et al., 1999]. RANK-L induces osteoclastogenesis by binding to RANK, a transmembrane receptor expressed on the surface of osteoclast progenitor cells [Burgess et al., 1999; Hofbauer et al., 2000; Lacey et al., 2000]. Osteoprotegerin is a decoy RANK-L receptor that is expressed on osteoblasts. Glucocorticoids have been shown to increase RANK-L expression and decrease osteoprotegerin expression on human osteoblasts *in vitro* [Hofbauer et al., 1999], thereby increasing osteoclastogenesis. Furthermore, glucocorticoids increase the expression of PTH receptors on osteoblasts, thereby increasing their sensitivity to PTH [Urena et al., 1994]. These effects would explain the proposed initial increase in resorption following glucocorticoid administration *in vivo* [Reid, 1997]. However, the dependence of

osteoclastogenesis on osteoblasts is also consistent with the eventual decrease in bone resorption seen *in vivo*, since glucocorticoids deplete osteoblast numbers.

Glucocorticoids have been reported to suppress the hypothalamic-pituitary-adrenal axis at multiple levels. Though a feedback inhibition mechanism, they reduce adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary, thereby inhibiting the synthesis and secretion of adrenal androgens [Ziegler and Kasperk, 1998]. Glucocorticoids have also been shown to inhibit gonadotropin secretion and reduce testicular and ovarian steroidogenesis [Patschan et al., 2001; Rodino and Shane, 1998]. A hypogonadal state may contribute indirectly to bone loss [Lukert and Raisz, 1990]. Estrogens and androgens are thought to inhibit the release of stimulating factors for osteoclast precursor formation by osteoblasts [Jilka et al., 1992; Weinstein et al., 1998a]. Thus a reduction in their levels serves to increase the number of osteoclasts available for bone resorption.

In summary, glucocorticoids may also influence bone metabolism through their effects on other organ systems. Such effects include changes in intestinal calcium absorption and renal calcium excretion, changes in parathyroid gland secretion of PTH, and changes in steroid synthesis by the adrenal cortex and by the gonads (Table 1.2). The responses of these organs in turn affect those cells that directly maintain bone function.

1.3 Osteoblastic differentiation *in vitro*

Osteoprogenitor cells (i.e., cells capable of differentiating into osteoblasts under the appropriate stimuli) have been identified in both animal and human bone marrow stroma [Friedenstein et al., 1987; Haynesworth et al., 1992; Maniatopoulos et al., 1988]. These cells are thought to be derived from a multipotent mesenchymal stem cell that has the capacity to differentiate into osteoblasts (bone), myocytes (muscle), tenocytes (tendon), chondrocytes (cartilage), adipocytes (fatty tissue), and hematopoiesis-supportive stromal cells [Beresford, 1989; Caplan, 1991; Owen, 1988]. Osteoblastic differentiation *in vitro* is marked by three distinct stages of cellular activity: proliferation, extracellular matrix maturation, and matrix mineralization [reviewed by Lian and Stein, 1992]. Initially, osteprogenitor cells are highly mitotic, as demonstrated by the expression of the cell-growth associated genes H4 histone and *c*-fos [Pockwinse et al., 1992]. During this period, genes associated with extracellular matrix formation (type I collagen, fibronectin, TGF-β) are expressed at peak levels. As proliferation

decreases, expression of the bone/liver/kidney isoform of alkaline phosphatase increases to peak levels. This enzyme is believed to have an important role in preparing the extracellular matrix for mineralization. Following a period of matrix maturation, nodule cells begin to mineralize the collagen-based extracellular matrix. The expression of osteocalcin and bone sialoprotein increase with mineral deposition, while alkaline phosphatase levels begin to decline.

The proliferation, differentiation, and metabolic activity of osteoblastic lineage cells are regulated by extracellular stimuli such as soluble factors, cell-cell interactions, and cell-matrix interactions. Local soluble factors, including IGFs and prostaglandins, regulate osteoblastic function through autocrine and paracrine mechanisms. Prostaglandin E_2 (PGE_2) enhances bone-like tissue formation in both marrow stromal cell [Scutt et al., 1995; Weinreb et al., 1999] and rat calvarial osteoblast [Kaneki et al., 1999; Nagata et al., 1994; Tang et al., 1996] cultures. Furthermore, administration of PGE_2 to both young [Weinreb et al., 1997] and aging [Keila et al., 2001] rats *in vivo* has been shown to increase the osteogenic capacity of marrow stromal cells when cultured *ex vivo*. Acting through G-protein-linked receptors, this eicosanoid has been reported to increase both synthesis of the secondary messenger cyclic adenosine 3',5'-monophosphate (cAMP) and accumulation of inositol trisphosphate (IP_3) in marrow stromal cells [Scutt et al., 1995] and the murine MC3T3-E1 cell line [Kozawa et al., 1993]. Reich and Fangos [1991] demonstrated that fluid shear-induced increases in cAMP and IP_3 levels were mediated by PGE_2 secretion. Thus this factor may play an important role in the response of osteoblasts to mechanical stimuli.

In addition to autocrine and paracrine signals, adjoining osteoblastic cells also communicate through intercellular channels known as gap junctions. These channels allow for the rapid transport of small molecules (e.g., calcium, cyclic nucleotides, inositol phosphates) from the cytoplasm of one cell to that of a connected cell [Donahue, 2000]. Gap junctions are comprised of two hexameric hemichannels (connexons), each of which is composed of six subunits known as connexins. Connexins 43, 45, and 46 have each been identified in bone cells [Koval et al., 1997; Steinberg et al., 1994]. However, connexin 43 is the predominate gap junction protein expressed in these cells. PTH and PGE_2 have been reported to stimulate gap junction-mediated communication by regulating connexin 43 expression and membrane localization through stimulation of cAMP within the adenylyl cyclase (AC) signal transduction pathway [Civitelli et al., 1998]. Lecanda et al. [1998] demonstrated, by altering the relative

expression of connexins 43 and 45 (increasing the ratio of 43 to 45) in URM 106-01 rat osteosarcoma cells, that gap junction permeability correlated with expression of the osteoblast-specific markers osteocalcin and bone sialoprotein. A similar study reported that the inhibition of gap junction-mediated communication in MC3T3-E1 cells caused decreases in alkaline phosphatase activity, matrix mineralization, and expression of both osteocalcin and bone sialoprotein [Schiller et al., 2001]. These studies suggest gap junctions play an important role in osteoblastic differentiation, and that intercellular communication may be necessary in coordinating the synthesis and mineralization of bone matrix [Schiller et al., 2001].

Cadherin-mediated cell-cell adhesion is important for the recruitment, proliferation, and differentiation of pre-osteoblasts at the site of new bone formation. Cadherins are single-chain integral membrane glycoproteins that have an amino-terminal extracellular domain, a single transmembrane domain, and a carboxy-terminal cytoplasmic tail [Kemler, 1992]. These proteins mediate gap junction formation and mechanical stability. Human osteoblasts express at least three cadherins: cadherin-2 (N-cad), cadherin-4 (R-cad), and cadherin-11 [Cheng et al., 1998]. These adhesion molecules may contribute to the recruitment of osteoprogenitors by providing molecular targets for migration toward the bone surface, as well as for differentiation [Cheng et al., 1998]. A recent study reported that specific blocking of N-cad expression decreased both matrix mineralization and the expression of bone matrix proteins (sialoprotein, osteocalcin, and type I collagen) [Cheng et al., 2000b].

Cell-matrix adhesion – primarily mediated by integrin receptors – is another important factor in bone cell development. Integrins are heterodimeric transmembrane glycoproteins composed of different combinations of two subunits (α and β). These receptors transduce signals from the extracellular matrix to adherent cells through specific signaling pathways (e.g., mitogen-activated protein kinase pathways) [Clark and Brugge, 1995]. Osteoblasts express multiple integrins, including $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ [Clover et al., 1992; Gronthos et al., 1997; Grzesik and Robey, 1994; Hughes et al., 1993; Saito et al., 1994]. These heterodimers have varying affinities for multiple bone matrix proteins such as osteopontin, bone sialoprotein, type I collagen, vitronectin, and fibronectin. Through interaction with integrins, bone matrix proteins may regulate the sequential differentiation of marrow stromal cells *in vitro* [Adams and Watt, 1993]. A previous study supported this hypothesis by demonstrating that the interaction between the $\alpha_5\beta_1$ dimer and fibronectin mediates the differentiation of fetal rat calvarial cells

[Moursi et al., 1997]. Similarly, the interaction between type I collagen and the α_2 subunit has been reported to be necessary for the expression of the osteoblastic markers in the murine MC3T3-E1 cell line [Xiao et al., 1998].

1.4 Effects of glucocorticoids on osteoblastic differentiation

Bone-like tissue has been synthesized *in vitro* from primary marrow stromal cells cultured in the presence of ascorbic acid, β-glycerophosphate, and the glucocorticoid dexamethasone [Cheng et al., 1994; Kasugai et al., 1991; Malaval et al., 1994; Maniatopoulos et al., 1988; Peter et al., 1998]. Dexamethasone (9α-fluoro-16α-methylprednisolone) is a synthetic analog of cortisol. In marrow stromal cell cultures, physiological concentrations of dexamethasone have been shown to produce multi-layered cell colonies that positively stain for alkaline phosphatase and develop into mineralized bone nodules [Aubin, 1999; Herbertson and Aubin, 1995; Jaiswal et al., 1997]. A morphological alteration has been observed in these cultures, in which elongated, fibroblastic cells were replaced with clusters of smaller, cuboidal cells [Cheng et al., 1994; Malaval et al., 1994; Maniatopoulos et al., 1988]. Increased levels of alkaline phosphatase activity [Cheng et al., 1994; Majeska et al., 1985; Maniatopoulos et al., 1988; Rickard et al., 1994] and cyclic adenosine 3',5'-monophosphate (cAMP) response to both PTH [Chen and Feldman, 1978; Cheng et al., 1994; Wong, 1979] and prostaglandin E_2 (PGE_2) [Cheng et al., 1994] have also been reported. Dexamethasone also enhances expression of markers for mature osteoblasts (e.g., osteocalcin and bone sialoprotein) [Aubin, 1999; Leboy et al., 1991; Kasugai et al., 1991; Rickard et al., 1994] and stimulates mineralization of the secreted extracellular matrix [Aubin, 1999; Cheng et al., 1994; Dieudonne et al., 1999; Maniatopoulos et al., 1988].

Glucocorticoids have been reported to affect the expression of osteoblastic proteins that regulate both cell-cell and cell-matrix interactions. First, dexamethasone suppresses N-cad and cadherin-11 mRNA transcription while stimulating cadherin-4 expression in both human trabecular osteoblast and human marrow stromal cell cultures [Lecanda et al., 2000]. Second, glucocorticoids may also alter connexin expression – affecting gap junction-mediated intercellular communication – through its effects on G-protein expression. G proteins are known to regulate the signaling between hormonal receptors and multiple effector pathways, including the adenylyl cyclase (AC) and phospholipase C (PLC) transduction pathways [Mitchell and

Bansal, 1997]. In ROS 17/2.8 rat osteosarcoma cells, dexamethasone is thought to increase PTH-stimulated AC activity by enhancing the expression of both $G\alpha_s$ and an associated PTH receptor [Rodan and Rodan, 1986]. In UMR-106-01 cells, dexamethasone was shown to increase $G\alpha_{q\text{-}11}$ protein and PTH-activation of the PLC signal transduction pathway [Cheung and Mitchell, 2002]. These studies suggest that dexamethasone may stimulate osteoblastic differentiation though changes in gap junction-mediated intracellular communication. Third, glucocorticoids alter the expression of proteins that regulate cell-matrix adhesion and signal transduction. Dexamethasone has been reported to down-regulate expression of the α_2 and α_4 integrin subunits in primary human marrow stromal cell cultures [Walsh et al., 2001]. These subunits – when heterodimerized with the β_1 subunit – are known to bind with type I collagen ($\alpha_2\beta_1$), osteopontin ($\alpha_4\beta_1$), and fibronectin ($\alpha_4\beta_1$) [Grzesik and Robey, 1994]. Furthermore, corticosterone has been reported to inhibit expression of the β_1 subunit in primary rat osteoblasts and in a rat osteosarcoma-derived cell line (ROS 17/2.8) [Gronowicz and McCarthy, 1995]. Using both mature human osteoblast and human marrow stromal cell cultures, Cheng et al. [2000a] demonstrated that long-term exposure to dexamethasone inhibited the expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins, known receptors for osteopontin and vitronectin.

Glucocorticoids have diverse and complex effects on bone cells. While pharmacological glucocorticoid doses are known to induce osteoporosis by decreasing bone formation *in vivo*, physiological concentrations of these steroid hormones have been shown to stimulate osteoblastic differentiation of progenitor cells *in vitro* [Bellows et al., 1987; Jaiswal et al., 1997; Maniatopoulos et al., 1988]. Furthermore, the reported effects of glucocorticoids *in vitro* have varied with the dosage and timing of treatment, the species/sex/age of the cell donor, the anatomical source of harvested progenitor cells (e.g., marrow stromal cells, calvarial cells), the maturation of the cells studied (i.e., the duration of primary and secondary cultures), the cell seeding density, and the combination of additional supplements in the culture medium. For example, at physiological concentrations dexamethasone has been reported to have inhibitory, stimulatory, or negligible effects on the proliferation of human- [Cheng et al., 1994; Kim et al., 1999; Walsh et al., 2001] and rat-derived marrow stromal cells [Beresford et al., 1992; Lennon et al., 1995; Milne et al., 1998; Scutt et al., 1996]. However, pharmacological levels of dexamethasone clearly inhibit osteoprogenitor cell proliferation [Kim et al., 1999; Scutt et al., 1996; Walsh et al., 2001]. Conflicting results have also been published regarding the effects of

glucocorticoids on collagen expression *in vitro*. Depending on the cell model and culture conditions, glucocorticoids have been reported to have either an overall stimulatory [Yao et al., 1994], overall inhibitory [Kim et al., 1999; Leboy et al., 1991], or a biphasic effect [Shalhoub et al., 1992] on type I collagen expression.

In addition to early markers of osteoblastic differentiation, differences in bone-like mineral deposition and expression of proteins associated with mineralized matrix have also been attributed to variations in culture conditions *in vitro.* For example, the maturity of the progenitor cells at the point of initial glucocorticoid supplementation influences the degree of osteoblastic differentiation. Multiple studies using rat marrow stromal cells have demonstrated that dexamethasone treatment in both primary and secondary culture enhances differentiation when compared with treatment in either passage alone [Aubin, 1999; Beresford et al., 1992; Ter Brugge and Jansen, 2002]. Similar findings have been reported in rat calvaria cell cultures [Bellows et al., 1987]. Furthermore, maximal osteocalcin and bone sialoprotein expression was measured with continuous dexamethasone supplementation [Atmani et al., 2002] of rat marrow stromal cells. Cell seeding strategy also affects differentiation *in vitro*. Previous studies that have demonstrated enhanced mineralization associated with increased osteoprogenitor seeding density [Aubin, 1999, Goldstein et al., 2001; Herbertson and Aubin, 1995; Jaiswal et al., 1997].

These variations in osteoblastic differentiation demonstrate a need to further investigate the particular mechanisms involved in glucocorticoid action. They also suggest that glucocorticoids such as dexamethasone are convenient but non-ideal stimulants for the osteoblastic differentiation of marrow stroma-derived progenitor cells.

1.5 Purpose of Study

The purpose of this study was to examine specific aspects of glucocorticoid action on bone metabolism. Specifically, there were three central aims to the experimental work:

1.) *To create and characterize a rat model for steroid-induced osteopenia and adipogenesis of the marrow stroma.*

Long-term exposure to pharmacological levels of glucocorticoids has been associated with the development of osteopenia, which is characterized by substantial trabecular bone loss, reduced bone strength, and increased incidence of fracture. High doses of glucocorticoids are also

thought to increase marrow adipogenesis. To create a model for osteopenia and marrow adipogenesis, Sprague-Dawley rats were treated with methylprednisolone for up to six weeks, then sacrificed at 0, 2, 4, or 6 weeks during treatment or 4 weeks after cessation of treatment. Cortical bone strength, mineral content, and fatty marrow volume were analyzed to assess the effects of steroid administration. This study is presented in Chapter 2.

2.) *To identify any changes in the osteogenic potential of bone marrow stroma in response to glucocorticoid treatment.*

Fibroblast-like progenitor cells in the bone marrow stroma may differentiate along several developmental pathways to form osteoblasts, chondrocytes, myocytes, tenocytes, adipocytes, and hematopoesis-supportive stromal cells. Pharmacological concentrations of glucocorticoids appear to concurrently undermine osteoblastic differentiation and stimulate adipocytic differentiation of these cells *in vivo*. Therefore, glucocorticoid administration may undermine the capacity of marrow stroma-derived osteoprogenitor cells to differentiate into osteoblasts. However, this effect may be reversible following discontinuation of treatment. To test these hypotheses, marrow stromal cells from methylprednisolone-treated rats were cultured *in vitro* in the presence of the osteogenic supplements β-glycerophosphate, ascorbate-2-phosphate, and dexamethasone. The osteogenic potential of marrow stromal cells was characterized by the expression and secretion of osteocalcin (a marker of mature osteoblasts) and formation of bone-like mineral. This study is presented in Chapter 3.

3.) *To examine the effects of short term glucocorticoid exposure on osteoblastic differentiation of marrow stromal cells cultured in vitro.*

Dexamethasone appears to be an effective but nonspecific inductor of osteoblastic differentiation within both rat and human bone marrow stromal cell cultures. However, this synthetic glucocorticoid has been shown to inhibit both osteoprogenitor cell proliferation and type I collagen expression in marrow stromal cell cultures. These findings suggest that continuous treatment with dexamethasone may undermine osteoblastic differentiation of the osteoprogenitor cells, and that removing dexamethasone after initial osteoinduction may enhance terminal osteoblastic differentiation. To test this hypothesis, osteoprogenitor cells – isolated from rat bone marrow and expanded in primary culture – were cultured in the presence of the osteogenic

supplements dexamethasone, ascorbate-2-phosphate, and β-glycerophosphate. Cell density, type I collagen synthesis, phenotypic marker expression, and bone-like mineral deposition were analyzed after varying duration of steroid supplementation. This study is presented in Chapter 4.

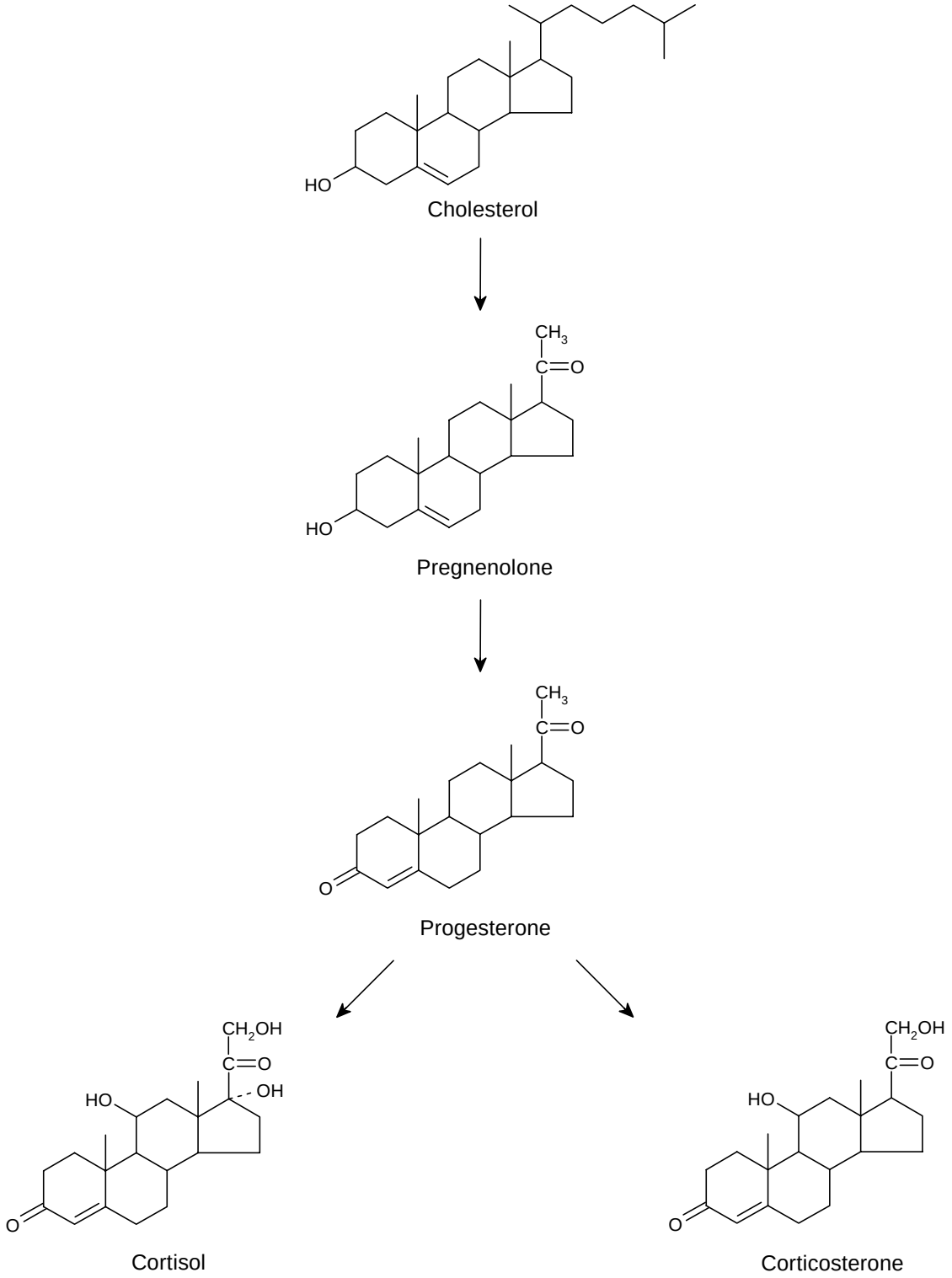

Figure 1.1: Biosynthesis of glucocorticoids from cholesterol.

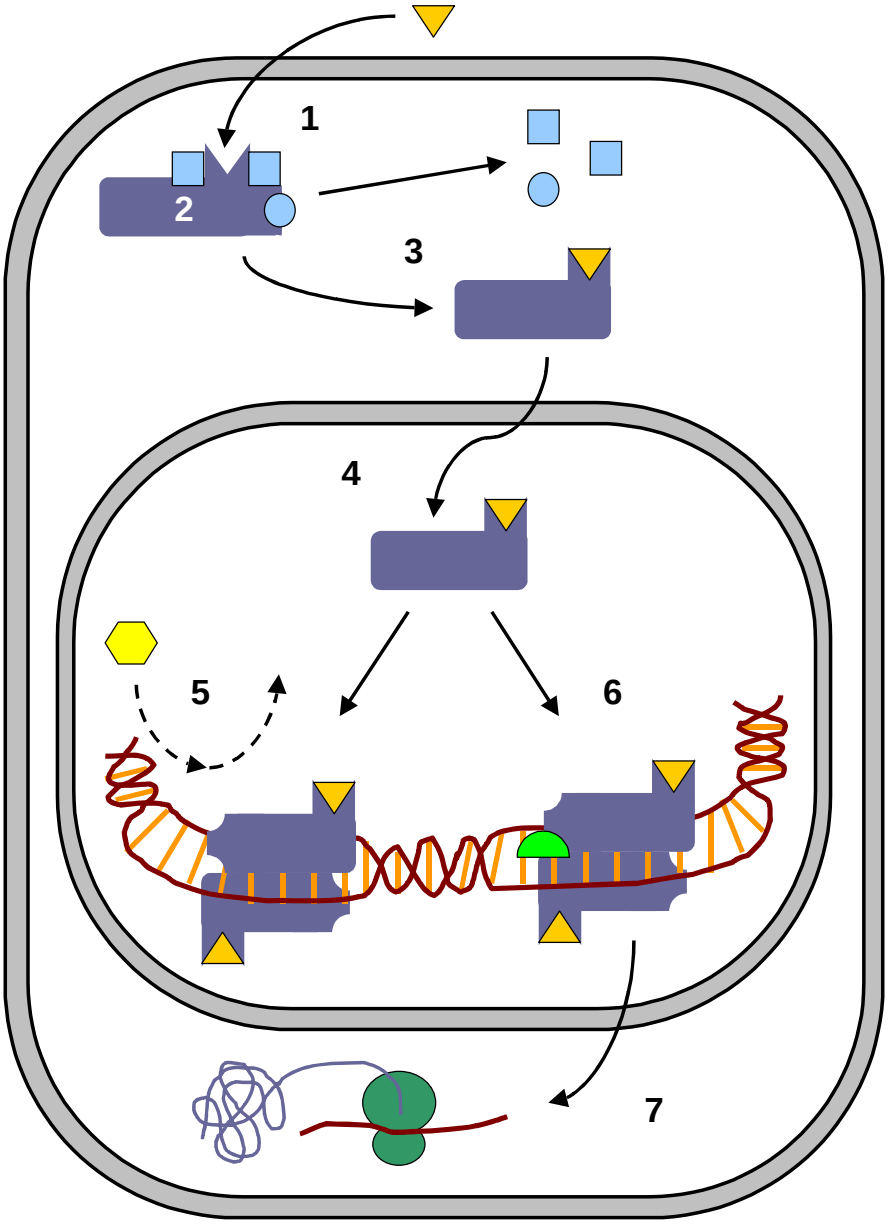

Figure 1.2: Regulation of transcription by glucocorticoids. (1) Extracellular glucocorticoid diffuses through the receptor membrane without the aid of a protein channel. (2) The cytosolic glucocorticoid receptor (cGR) is retained in the cytoplasm as part of a complex with molecular chaperones (light blue). This conformation has a high affinity for steroid. (3) Upon binding with its ligand, the cGR dissociates from the chaperone proteins. (4) Now in an active state, the steroid-receptor complex translocates into the nucleus. (5/6) The cGR dimerizes and binds with cognate DNA sequences (glucocorticoid reponse elements) near target gene promoters. The bound cGR dimer may either (5) hinder the binding of other transcription factors or (6) facilitate their interaction with promoter sites, (7) facilitating transcription and subsequent protein translation. The cGR may also inhibit transcription by directly binding with transcription factors, thus blocking their activity (not shown).

Depleted osteoblast numbers:
Decreased osteoprogenitor cell recruitment
Decreased preosteoblast proliferation
Increased osteoblast apoptosis
Altered osteoblast metabolism:
Decreased IGF-I secretion
Decreased IGFBP-5 synthesis
Decreased type I collagen synthesis

Table 1.1: Effects of glucocorticoids on bone formation.

Calcium depletion:
Decreased intestinal calcium absorption
Increased renal calcium excretion
Altered PTH secretion?
Enhanced osteoclastogenesis (early):
Increased RANK-L expression
Decreased osteoprotegerin expression
Decreased estrogen levels
Decreased adrenal androgen levels
Diminished osteoclastogenesis (late):
Depleted osteoblast numbers (see Table 1.1)
Increased collagenase secretion by osteoblasts

Table 1.2: Effects of glucocorticoids on bone resorption.

Chapter 2:

Evaluation of a rat model for glucocorticoid-induced osteopenia and bone marrow adipogenesis

2.1 Introduction

Glucocorticoids are administered for the treatment of various inflammatory disorders and to prevent organ transplant rejection. However, exposure to pharmacological doses of these steroid hormones has been associated with the development of osteopenia, which is characterized by substantial trabecular bone loss and increased risk of fracture [reviewed by Canalis, 1996; Reid, 1997]. Glucocorticoid-induced bone loss can been primarily attributed to a decrease in bone formation [Dempster, 1989], consistent with an alteration in the number or metabolic activity of osteoblasts. In addition to osteopenia, glucocorticoids have also been reported to increase the volume of marrow fat in humans [Vande Berg et al., 1999] and in animals [Kwai et al., 1985, Wang et al., 1977]. By increasing intraosseous pressure within the marrow compartment, an elevation in fatty marrow volume may lead to avascular necrosis within trabecular bone [Wang et al., 1977]. Both of these conditions may be partially attributed to glucocorticoid-induced changes in osteoblastic and adipocytic differentiation.

Osteoblasts, adipocytes, and other connective tissue phenotypes are derived from a heterogeneous population of progenitor cells within the bone marrow stroma [Beresford, 1989; Caplan, 1991; Owen, 1988]. Previous studies have demonstrated that osteoblasts and adipocytes share a common precursor cell within this population [Bennett et al., 1991] and that the degrees of osteogenesis and adipogenesis are reciprocal within marrow stroma-derived cell cultures [Beresford et al., 1992; Cui et al., 1997]. Furthermore, Cui et al. [1997] reported that dexamethasone stimulated adipogenesis and concurrently suppressed osteogenesis in a dose-dependent manner. Therefore, the correlation between suppressed bone formation and increased marrow fat during glucocorticoid administration *in vivo* may indicate that these steroids inhibit the osteoblastic differentiation of progenitor cells by directing their differentiation into adipocytes. By depleting the number of osteoblasts available for bone remodeling and repair, this shift in progenitor cell differentiation may contribute to the pathogenesis of osteopenia and avascular necrosis [Nuttall and Gimble, 2000].

The aim of this study was to create a model for glucocorticoid-induced osteopenia and adipogenesis in order to identify any changes in the capacity of marrow stromal progenitor cells to differentiate into mature osteoblasts (i.e., their osteogenic potential) when cultured in the presence of osteogenic supplements *in vitro* (as described in Chapter 3). We hypothesize that pharmacological levels of glucocorticoid will reduce cortical bone strength, decrease bone mineral density, and increase the volume fraction of fatty marrow in the femoral diaphysis. These effects should be more pronounced with increasing duration of treatment; however, we anticipate some recovery of normal bone properties following cessation of treatment. The Sprague-Dawley rat was chosen as the animal model for this study: the behavior of rat marrow stromal cells have been extensively characterized *in vitro* [Leboy et al., 1991; Malaval et al., 1994; Maniatopoulos et al., 1988; Rickard et al., 1994], and their behavior is comparable with that of human marrow stromal cells cultured *in vitro* [Haynesworth et al., 1992; Cheng et al., 1994; Kim et al., 1999]. Furthermore, the deleterious effects of glucocorticoid excess on bone have been demonstrated in rat models [Li et al., 1996; Ørtoft and Oxlund, 1996].

Sprague-Dawley rats were administered methylprednisolone for up to six weeks, then sacrificed at 0, 2, 4, or 6 weeks during treatment or 4 weeks after cessation of treatment. Femurs were collected and analyzed for evidence of steroid-induced osteopenia and bone marrow adipogenesis. Cortical bone strength of rat femurs was analyzed using a shear test. Inorganic weight fraction was measured by thermogravimetric analysis of samples from the proximal femora. The fraction of fatty marrow within cross sections of the femoral mid-diaphysis was measured by histological analysis.

2.2 Materials and Methods

2.2.1 *Animal protocols*

Nineteen juvenile female Spraque-Dawley rats (175-200 g; Harlan, Dublin, VA) were housed, treated, and sacrificed at the Lab Animal Resources (LAR) facility using university-approved protocols. Two rats were kept per cage – except for a single cage of three – in a 25°C, 12-hr light / 12-hr dark environment. Rats were provided Purina rat chow and water *ad libitum*. After a week in quarantine, two rats from the same cage were sacrificed by CO_2 inhalation. The remaining 17 rats underwent one of two different treatment regimens. One animal from each cage received 18 mg/kg/day methylprednisolone (40 mg/mL in 8% benzyl alcohol; Pharmacia &

Upjohn, Kalamazoo, MI) by oral gavage, while the other underwent sham treatment. Oral gavage is a drug delivery technique that utilizes a special type of needle with a rounded end. Such a needle may be inserted through the esophagus and into the stomach without puncturing these organs. Rats were weighed daily to determine the methylprednisolone dose and to identify time-dependent trends in weight. The test animals received steroid for up to 42 consecutive days. Two methylprednisolone-treated animals and two control animals were sacrificed on days 14, 28, and 42. Test/control pairs were sacrificed from the same cages to minimize any anxiety due to changes in their environment. After 42 days, daily treatment with methylprednisolone was discontinued to allow for potential recovery of normal bone properties. The three remaining treated rats and the two controls were sacrificed on day 70.

Animal sacrifice and bone extraction took place in the necropsy room of the LAR facility. For each animal, the hind legs were exarticulated at the hip joints. Two tibias were placed into a 50 mL centrifuge tube (Fisher, Pittsburg, PA) containing 25 mL Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 10% antibiotic/antimycotic (penicillin, streptomycin, neomycin, and fungizone; Gibco BRL). One femur – with musculature still attached – was wrapped in gauze pre-soaked with phosphate buffered saline (PBS; Gibco BRL) and placed in a 50 mL centrifuge tube for mechanical testing. The other femur was gently scraped clean using the scalpel blade and placed in a 50 mL centrifuge tube containing 30 mL neutral-buffered formalin (NBF; Fisher) for histological analysis. The animal carcasses were disposed of according to LAR procedures.

2.2.2 Histological analysis of femurs

The femurs stored in NBF were decalcified in TBD solution (Thermo Shandon, Pittsburg, PA) for five days. After decalcification, two sections of the cortical diaphysis (midshaft) – each ~3 mm in length – were cut from the femur and embedded in paraffin. Five μm cross sections of each embedded diaphyses pair were mounted onto 3"×1" glass slides and stained with hematoxylin and eosin.

Stained tissue cross-sections were examined for areas of fatty and non-fatty marrow within the medullary compartment. One diaphysis cross section per slide – the one with fewer artifacts – was chosen for histological analysis. Images from the cross section were captured at

10X magnification on an Olympus model IX50 inverted microscope equipped with a Prior motorized stage accessory and a Coolpix 995 color digital camera (Nikon, Melville, NY). The captured images were tiled using ImagePro software (Media Cybernetics, Silver Spring, MD) to create a composite for the entire cross section. A histogram segmentation toolbox within ImagePro was then used to distinguish between pixels representing areas of bone, fatty marrow, and non-fatty marrow. Neglecting relatively large objects (i.e., due to artifacts from the sectioning process), adipocyte pockets within the marrow were counted and measured (Appendix B-3). Object areas were exported to Excel, and the areas of adipocytic and non-adipocytic marrow were summed for each cross section. Area fractions of fatty marrow were calculated from these totals.

2.2.3 *Mechanical testing of femurs*

The mechanical properties of explanted rat femurs were determined by a shear test as previously described in literature [Wilson et al., 2001; ASAE Standard S459 (1998)]. These bones, which had been stored at –20°C (in PBS-soaked gauze), were thawed at room temperature and the surrounding musculature was removed by gentle scraping with a scalpel blade. Bone dimensions were measured at the mid-diaphysis using a digital caliper. Femoral cross-sections were assumed to be hollow circular sections. The exterior bone diameter was determined by averaging the largest and smallest diameters at the mid-diaphysis prior to mechanical loading. After loading and shear (as described below), average wall thickness was estimated from three measurements at the shear surface.

Shear tests were performed using a MTS universal-testing machine (MTS Systems, Eden Prairie, MN) at a loading rate of 20 mm/min. Femurs were placed onto a double shear block apparatus [Wilson et al., 1984; ASAE Standard S459 (1998)] with the anterior side facing down (Figure 2.1). Shear force was exerted over a 12.7 mm length of the mid-diaphysis (i.e., the distance between the twin supports). Data acquisition was performed using TestWorks for Windows (MTS Systems), a software package specifically designed for materials testing. Output from TestWorks included peak load to fracture (N), peak stress to fracture (N/mm^2), and energy to peak load (N·mm). Following analysis, sheared femurs were re-wrapped in gauze and stored at –20°C.

2.2.4 *Thermogravimetric analysis of femurs*

The relative fractions of organic and inorganic mass in femoral bone were examined using thermogravimetric analsyis (TGA). The proximal half of the femurs used in shear testing were ground up with a mortal and pestle. The bone and marrow were separated by vigorous agitation in water. The bone fragments were dried overnight on Whatman filter paper, collected into microcentrifuge tubes, and stored at 4°C until analysis. A 10 mg sample from each bone was loaded onto a Pyris Thermogravimetric Analyzer (Perkin Elmer, Shelton, CT) and heated according to the following thermal program: 50°C for 1 min; 50 to 110°C at 20°C/min; 110°C for 30 min; 110 to 600°C at 10°C/min. The weight of the sample was recorded as a function of temperature and program time using Pyris software (Perkin Elmer). The weight fraction attributed to inorganic material was estimated by calculating the difference between the weight following incubation at 110°C (dry bone) and the weight at 600°C (ash), then dividing this difference by the initial sample weight. Similarly, the weight fraction attributed to inorganic material was estimated by dividing the weight at 600°C by the initial weight.

2.2.5 *Statistics*

Each measurement is presented as the mean ± standard deviation for rats given a similar treatment regimen. Unless otherwise noted, one response was measured per rat. A simple linear regression model was used to test for time-dependent trends over days 0–42 or days 0–70 (null hypothesis: slope = 0 for a linear model). A one-way analysis of variance in combination with Scheffe's multiple-comparison procedure was used to perform discrete pairwise comparisons of the means between treatment regimens. Statistical significance denotes a confidence of greater than 95% (α=0.05). Statistical analyses were performed using The SAS System for Windows release 8.02 (SAS Institute, Cary, NC), as described in Appendix A.

2.3 Results

2.3.1 Effect of methylprednisolone treatment on rat weight

Rats were given 18 mg/(kg body weight) methylprednisolone daily for a period of six weeks, then allowed to recover for an additional four weeks without any steroid. A marked decrease in weight was observed for the dosed rats immediately following the onset of

glucocorticoid administration (Figure 2.2). Following this initial decrease, the weights of the dosed animals did not significantly change during the four weeks of steroid treatment. However, the sham rats did gain a significant amount of weight during this period. Thus, the weight difference between treated and untreated groups increased during the four-week treatment period. After discontinuation of steroid treatment, dosed animal weights increased to the point where they were not significantly lower than the control group. Similar results were seen for both animal studies (Figure 2.2a and 2.2b).

2.3.2 Effect of methylprednisolone on the biomechanical properties of femurs

Animal femurs were subjected to a shear test in order to determine the effects of methylprednisolone treatment on mechanical properties of the cortical bone. Prior to testing, the mean periosteal (outer) diameter at the mid-diaphysis was measured for each femur. Periosteal diameters were significantly lower for dosed animals compared to controls after 42 days of treatment (Figure 2.3a). These diameters were still below control levels following the period of recovery ($p<0.05$). After mechanical failure, mean wall thickness was measured at the shearing surface. No significant differences in thickness were observed between the treated and control groups during either study (Figure 2.3b). Load measurements were collected as a function of bone deformation. The peak load at failure was significantly lower for the dosed animals after 28 days of treatment ($p<0.05$), but not following the recovery period (Figure 2.4a). By normalizing the peak load by estimated cross-sectional area (assuming a cylindrical geometry), the peak stress at failure (i.e., ultimate shear stress) was determined. There were no significant differences in the peak stress between the dosed and sham populations (Figure 2.4b).

2.3.3 Effect of methylprednisolone on the weight distribution of femoral bone

Sheared femurs from animals sacrificed at day 42 were tested for relative fractions of organic and inorganic mass by thermogravimetric analysis (TGA). Sample weight was relatively stable following the incubation at 110°C, indicating that the moisture had all been driven from bone fragments (Figure 2.5a). The subsequent decrease in sample weight had appreciably leveled of at 600°C. Therefore the sample weight at these points were used to determine fractions of inorganic material (i.e., mineral deposit) and organic material (i.e., matrix proteins). The weight fractions of inorganic material were estimated to be 0.177 ± 0.003 and 0.174 ± 0.003

for the treated and untreated rats, respectively, while the organic weight fractions were determined to be 0.729 ± 0.003 and 0.730 ± 0.011 (Figure 2.5b). Thus, no statistically significant differences were seen in either organic or inorganic fractions for the day 42 rats. Furthermore, no clear shifts were observed in the inflection points of the weight versus temperature plot, which might indicate a shift in the composition of individual bone matrix components.

2.3.4 Effect of methylprednisolone on fatty marrow content

Cross-sections of the femoral diaphysis were stained with hematoxylin and eosin for histological analysis. Tiled images were captured of these sections and analyzed for areas of fatty and non-fatty marrow. Adipose tissue appears as circular or oval-shaped pockets within the non-fatty marrow (Figure 2.6a). The area fraction of fatty marrow increased within treated rats from approximately 0.068 ± 0.037 at day 0 to 0.300 ± 0.066 at day 42 (Figure 2.6b). For the control rats, however, there was not a systematic change in the area of adipocyte pockets with increasing treatment duration. Thus the area fraction for the treated animals was significantly higher than the sham population (0.135 ± 0.040) after four and six weeks of treatment ($p<0.01$). This difference is apparent in the representative cross sections from day 28 rats (Figure 2.6a). Area fractions for treated animals decreased to levels similar to their sham counterparts after discontinuation of treatment.

2.4 Discussion

In this study, juvenile female rats were treated with pharmacological levels of methylprednisolone in order to produce an animal model for glucocorticoid-induced osteopenia and marrow adipogenesis. This treatment restricted both weight gain and radial bone growth when compared to controls. However, no differences were seen in either the ultimate shear stress or the inorganic weight fraction of femoral bone, suggesting that steroid treatment did not induce an appreciable osteopenic condition in this rat model. Conversely, an increase in the area fraction of fatty marrow was observed in these rats, indicating an enhancement of marrow adipogenesis by glucocorticoid treatment.

Steroid treatment has been shown to reduce food consumption and consequently weight gain in rats [Li et al., 1996; Ørtoft and Oxlund, 1988; Ørtoft et al., 1992]. In this study, food

consumption was not regulated in the control groups, thus weight gain was decreased in treated rats compared to controls. However, inanition (semi-starvation) does not appear to be the sole contributor to glucocorticoid-induced changes in cortical bone properties. A previous study with male Wistar rats demonstrated that 90 days of subcutaneous injection with 1 mg/kg/day methylprednisolone reduced the ultimate bending stress of the femoral diaphysis (an indicator of bone quality) when compared to food-restricted rats of the same age and weight [Ørtoft and Oxlund, 1988]. Conversely, food restriction induced a similar loss of ultimate bending strength when compared with subcutaneous injection of 5 mg/kg/day prednisolone (a glucocorticoid with protracted effect) for 80 days in female Wistar rats [Ørtoft et al., 1995]. However, food restriction did not increase the area fraction of osteocytic lacunae in cortical bone (a marker of diminished osteoid mineralization around the osteocytes), which was observed in prednisolone-treated rats [Ørtoft and Oxlund, 1996].

Pharmacological levels of methylprednisolone were shown to inhibit bone growth (in the radial direction) as indicated by outer diameter measurements at the femoral mid-diaphysis. There was no significant difference in bone wall thickness – as measured at the shear surface – between the treated and control populations. In rats, newly formed cortical bone is laid down as concentric lamellae on the periosteal (exterior) surface, while bone resorption occurs on the endosteal (interior) surface [Currey, 1984; Ørtoft and Oxlund, 1996]. The decrease in radial bone growth might be attributed to a glucocorticoid-regulated decrease in bone formation at the periosteal surface. Furthermore, the lack of change in wall thickness could indicate that osteoclast resorption also is inhibited by glucocorticoid administration. However, large variations in the measured wall thickness may have masked any subtle effects of glucocorticoid treatment on this property. These variances may be attributed to variability in the location of the shearing plane along the diaphysis. Alternatively, the decrease in radial bone growth might be attributed to a methylprednisolone-induced catabolic state within the rats [Ørtoft and Oxlund, 1995]. In contrast to our study, Ørtoft and Oxlund [1996] determined that prednisolone administration nearly arrested bone formation at the periosteal surface while increasing resorption at the endosteal surface, which resulted in a net decrease in cortical bone wall thickness.

Femurs from methylprednisolone-treated rats were examined using a shear test and shown to fail at lower ultimate loads when compared to controls. After correcting for differences

in cross-sectional area, however, there were no significant differences in ultimate shear stress between the treatment and control groups. These results do not support the presence of a qualitative change in the mechanical properties of the cortical bone, which might result from an accumulation of microdamage, an increase area fraction of osteocytic lacunae, or an increase in the number of endosteal resorption cavities. In contrast, the earlier study by Ørtoft and Oxlund [1988] demonstrated that the ultimate bending stress was significantly lower after 90 days of subcutaneous injection with 1 mg/kg/day methylprednisolone treatment than for both sham and saline-treated controls. However, no differences in cortical bone strength were observed after only 30 days of treatment when compared to control groups, suggesting that changes in the mechanical properties of cortical bone are more dependent on the duration of glucocorticoid treatment than on the cumulative dose.

Thermogravimetric analysis of ground femur samples indicated that there were no difference in the mass fractions of water, matrix proteins (e.g., collagen), or deposited mineral within rats treated with glucocorticoid for 42 days. These results support the shear test findings, suggesting that there is no change in bone mineral density or gross architecture of the cortical bone from treated rats. Ørtoft and Oxlund [1996] demonstrated that prednisolone treatment increased porosity in mid-diaphyseal cross sections by increasing the number of endosteal resorption cavities and the osteocytic lacunae area fraction. The increase in porosity positively correlated with a decrease in ash weight of cortical bone cylinders from the same rats [Ørtoft and Oxlund, 1996]. The authors attributed the decrease in ultimate bending stress reported in a previous study [Ørtoft and Oxlund, 1995] to this increase in resorption cavities, citing the correlation between decreased bone strength and increased porosity in both human and bovine cortical bone [Evans and Vincentelli, 1974; Martin and Ishida, 1989]. For our model, the lack of change in bone strength and inorganic weight fraction further demonstrates a possible inhibition of cortical bone resorption by methylprednisolone treatment. In human bone, the inorganic and organic components make up approximately 65% and 25%, respectively, of the bone matrix [Buckwalter et al., 1996a]. The differences between these literature values and the measured values from this experiment may indicate that the samples need to be held at 600°C for a longer duration.

Trabecular bone was not characterized in this rat model. Glucocorticoid treatment affects regions of trabecular bone to a larger extent than regions of cortical bone, due to the higher rate

of metabolism in trabecular bone [Adinoff and Hollister, 1983; Rickers et al., 1984]. Therefore, an analysis of the trabecular bone may demonstrate osteopenic characteristics within the methylprednisolone-treated rats.

A direct comparison of rat and human bone is limited, as significant physiological differences exist between the species. Specifically, rat cortical bone has a circumferential lamellar structure, while human cortical bone is composed of Haversian systems, or osteons, consisting of concentric lamellae surrounding a central canal [Ørtoft and Oxlund, 1988]. Thus rat bone is not remodeled by the same mechanism as is human bone. Such distinctions should be considered when applying the results of any animal model to the pathology of osteopenia in human bone.

Histological analysis of diaphyseal cross-sections demonstrated an increase in the volume fraction of adipocyte pockets in the marrow from steroid treated rats. The results suggest that pharmacological glucocorticoid levels increase fatty (yellow) marrow volume in the rat femora. Previous studies of osteonecrosis in humans [Vande Berg et al., 1999] and in rabbit models [Kwai et al., 1985; Wang et al., 1977] have associated an increase in yellow marrow with glucocorticoid administration. We cite two mechanisms for the increase in bone marrow fat that are not mutually exclusive. First, steroid treatment may cause an increase in fat cell volume (adipocyte hypertrophy) due to increased lipid synthesis and vesicle deposition. A recent study reported fat cell enlargement and a corresponding increase in intraosseous pressure in rabbits treated with methylprednisolone [Miyanishi et al., 2002]. Second, steroid treatment may enhance marrow adipogenesis. The progenitor cells for adipocytes, osteoblasts, and other connective tissue phenotypes are located within the bone marrow stroma [Beresford, 1989; Owen, 1988; Caplan, 1991]. Using a cell line derived from the mouse marrow stroma, Cui et al. [1997] reported that dexamethasone treatment stimulated adipogenesis of the culture-adherent progenitor cells in a dose-dependent manner, while concurrently suppressing osteoblastic differentiation.

Supra-physiological glucocorticoids levels may deplete osteoblast numbers *in vivo* by directing their precursors to differentiate into adipocytes [Cui et al., 1997]. This mechanism may be important in the development of glucocorticoid-induced osteoporosis [Cui et al., 1997; Nuttall and Gimble, 2000]. In addition to altering the relative rates of osteoblastic and adipocytic differentiation, however, these steroid hormones may irreversibly alter the capacity of marrow

stromal progenitor cells to differentiate into mature osteoblasts (i.e., their osteogenic potential). Glucocorticoids might alter the osteogenic potential of this cell population either by diminishing their number or by making these cells less responsive to osteoinductive stimuli. To identify such changes in glucocorticoid-treated rats, marrow stromal cells were isolated and cultured *ex vivo* in the presence of osteogenic factors (described in Chapter 3).

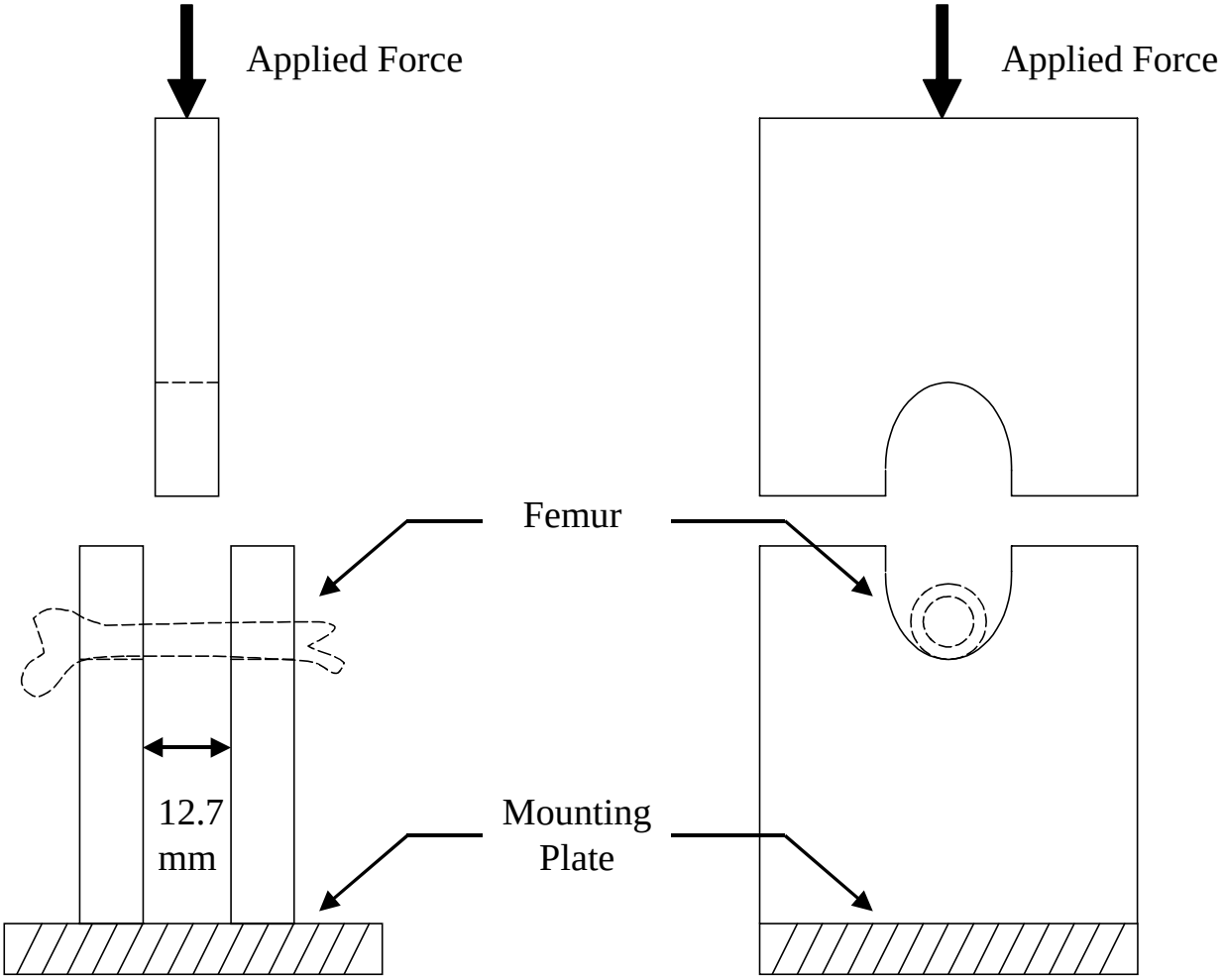

Figure 2.1: Diagram of double shear block apparatus.

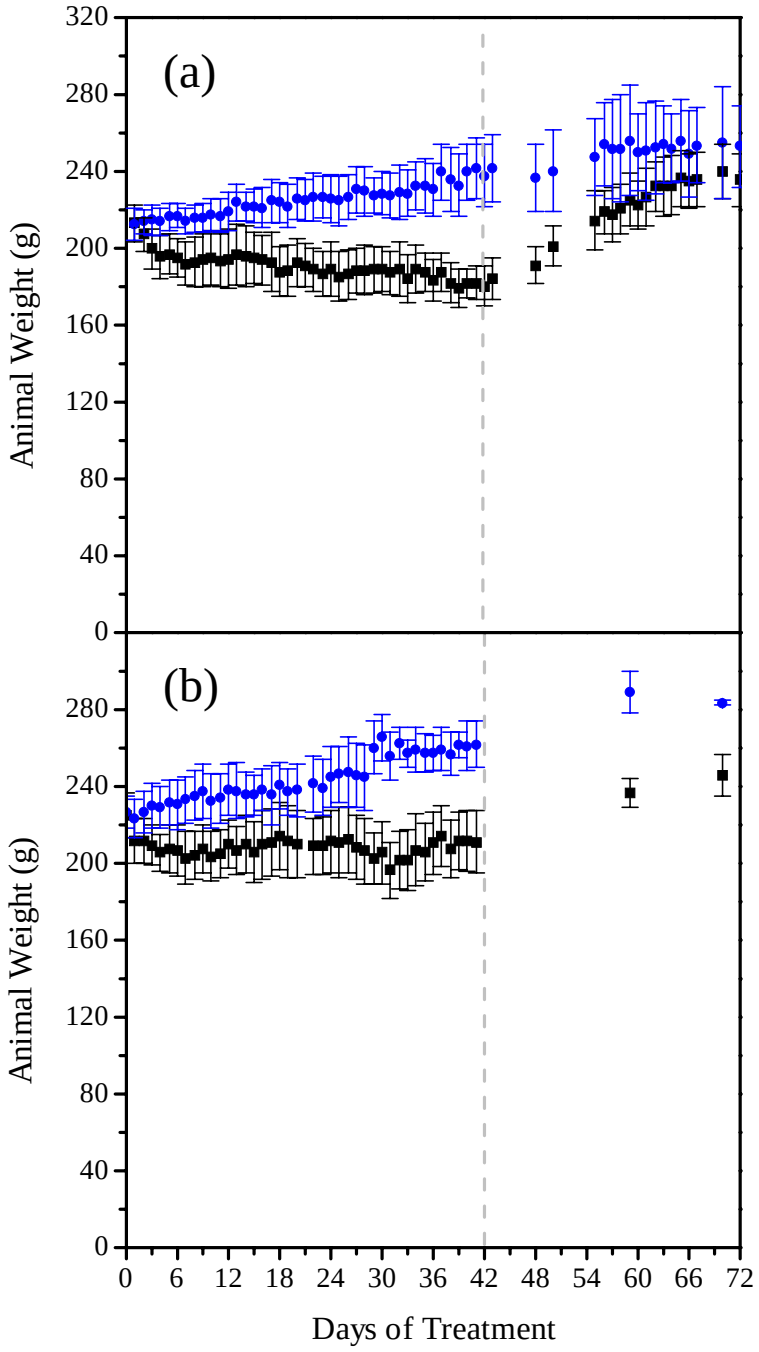

Figure 2.2: Rat weights throughout study. Mean animal weights for dosed (squares) and sham (circles) rats are plotted as a function of methylprednisolone treatment duration. Error bars initially correspond to the standard deviation of n = 9 treated rats and n = 8 sham rats. The number of animals decreases by 2 (for both populations) at days 14, 28, and 42. Group numbers are the same for the first (a) and second (b) animal studies. Treatment was discontinued at day 42 for the remaining rats (3 treated, 2 sham). The treatment and recovery periods are divided by the dashed line.

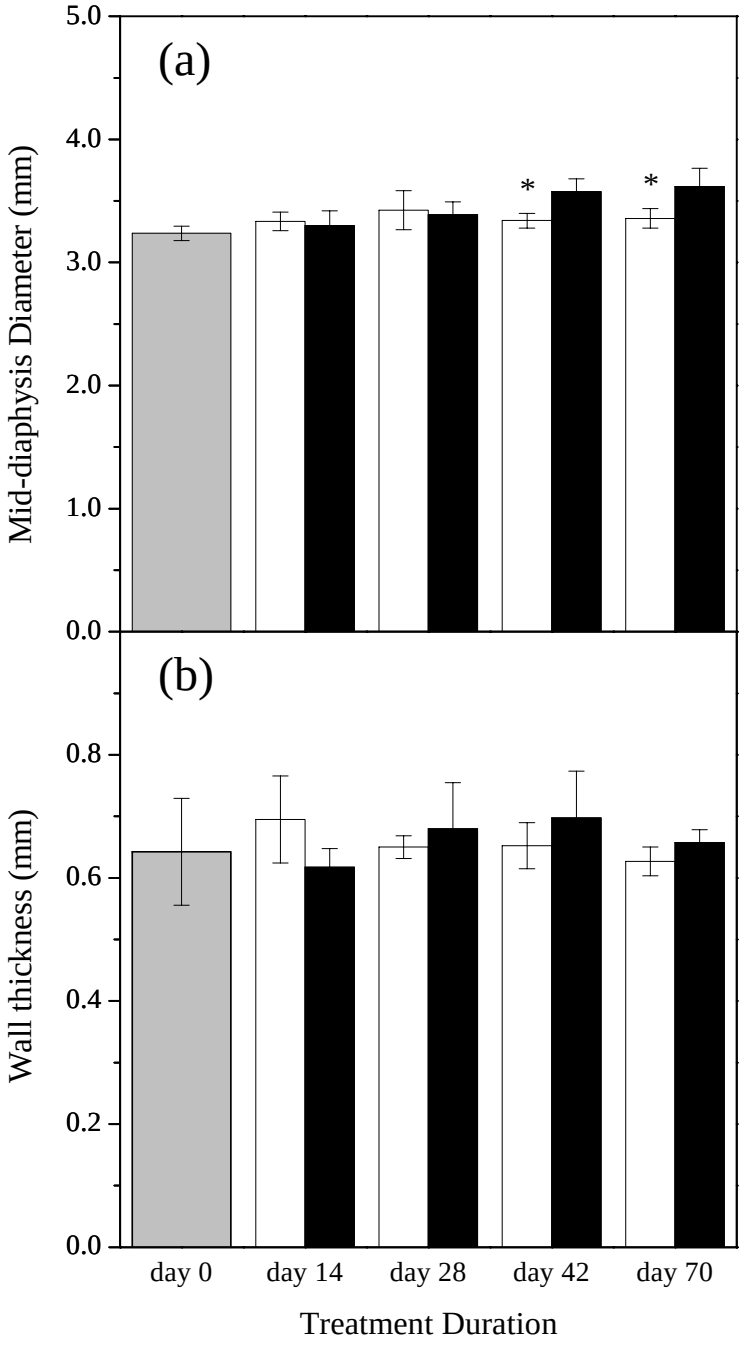

Figure 2.3: Dimensions of femoral mid-diaphysis. The mean diameter (a) and mean wall thickness (b) at the mid-diaphysis for femurs explanted from treated (white), sham (black), or day 0 control (gray) rats are plotted as a function of methylprednisolone treatment duration. Error bars correspond to the standard deviation of n = 4 (n = 5 for white bar, day 70) femurs (one per animal). An asterisk (*) denotes a statistically significant difference in periosteal diameter between treated and untreated rats (at a single timepoint).

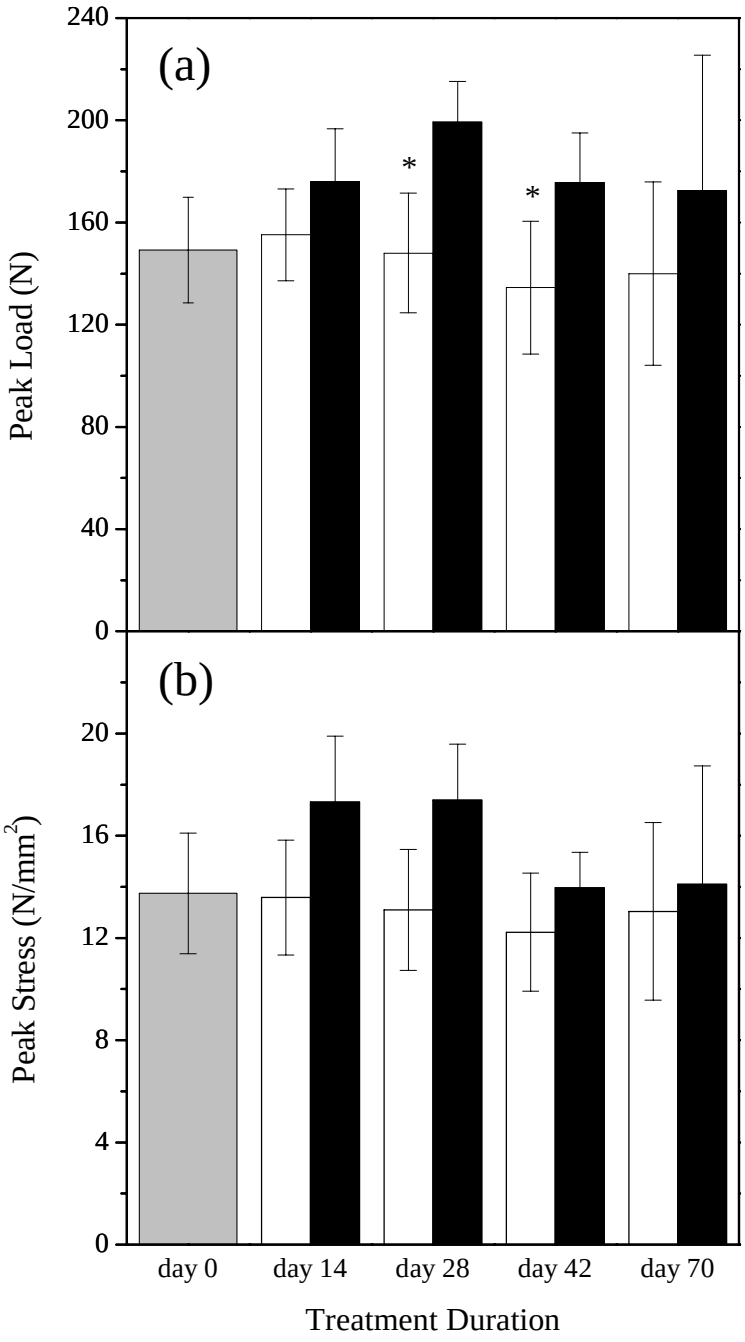

Figure 2.4: Mechanical properties of femurs. The peak load at failure (a) and the peak stress at failure (b) for femurs explanted from treated (white), sham (black), and day 0 control (gray) rats are plotted as a function of treatment duration. Error bars correspond to the standard deviation of n = 4 femurs, except for day 0 (n = 3), day 14 sham (n = 3) and day 70 treated (n = 5). An asterisk (*) denotes a statistically significant difference in ultimate load between treated and untreated rats (at a single timepoint).

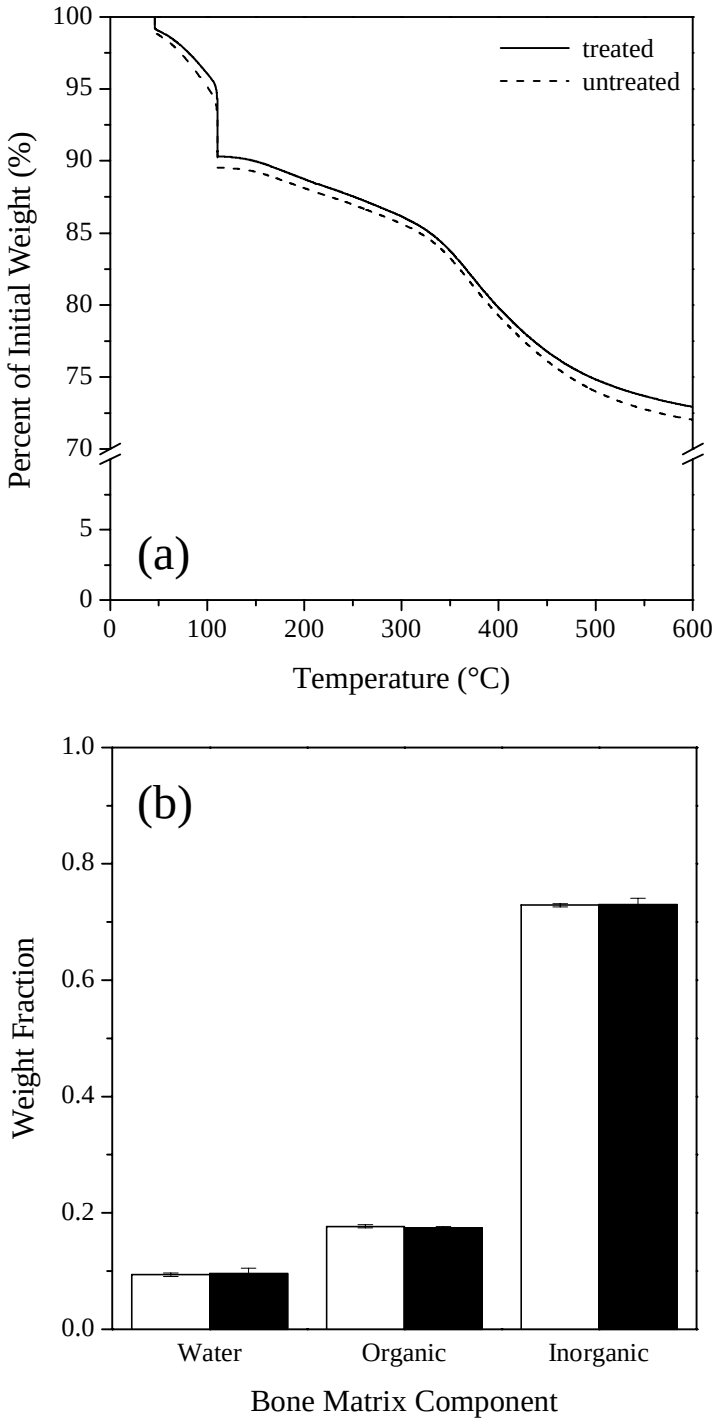

Figure 2.5: Weight distribution of femurs as determined by TGA. (a) The percent sample weight remaining during the heating process is plotted against sample temperature for samples from two representative day 42 rats. (b) The mean weight fractions of moisture, organic material (i.e., matrix proteins), and inorganic material (i.e., mineral deposits) are presented for dosed (white) and sham (black) animals. Error bars correspond to the standard deviation of n = 4 femur samples (one per animal).

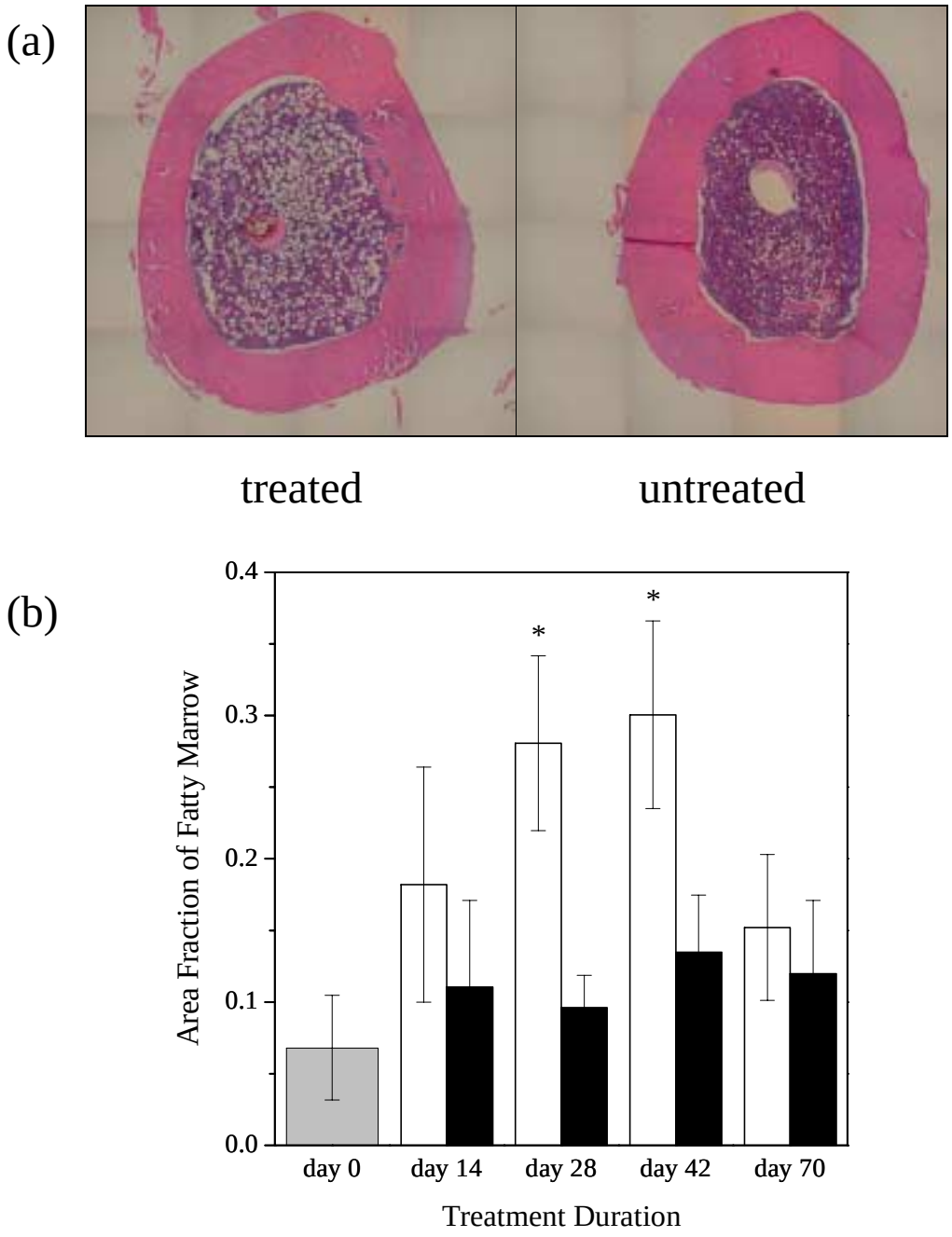

Figure 2.6: Marrow adipose tissue content in cortical diaphysis. (a) Differences in the marrow from treated and untreated rats at day 28 are illustrated in representative mid-diaphyseal cross sections. (b) Area fractions of adipose tissue within femoral diaphysis cross-sections from treated (white), sham (black), and day 0 control (gray) rats are plotted versus methylprednisolone treatment duration. Error bars correspond to the standard deviation of n = 4 (n = 5 for white bar, day 70) cross-sections (one per animal). An asterisk (*) denotes a statistically significant difference in area fraction of fatty marrow between treated and untreated rats (at a single timepoint).

Chapter 3:

Effects of glucocorticoid treatment in vivo on the osteogenic potential of rat bone marrow stromal cells in vitro

3.1 Introduction

The bone marrow stroma is a heterogeneous network of cells that provide structural and instructional support for hematopoiesis, including fibroblasts, endothelial cells, reticulocytes, and adipocytes. This connective tissue is also a repository for culture-adherent, fibroblast-like progenitor cells that are capable of differentiating into osteoblasts, adipocytes, chondrocytes, hematopoeisis-supportive stromal cells, and other mesenchymal phenotypes [Beresford, 1989; Caplan, 1991; Owen, 1988]. This progenitor cell population has a complex hierarchy, consisting of putative stem cells and their lineage-committed progeny [Caplan, 1994]. Studies using marrow stroma-derived cell cultures suggest that osteoblasts and adipocytes share a common precursor [Bennett et al., 1991] and that the relationship between osteogenesis and adipogenesis is reciprocal within the marrow stroma [Beresford et al., 1992]. Therefore, an increase in marrow adipogenesis may concurrently decrease the population of osteoblasts available for bone remodeling.

A correlation between bone loss and increased volume of marrow adipose tissue has been demonstrated in patients afflicted with osteoporosis and age-associated osteopenia [Burkhardt et al., 1987; Meunier et al., 1971], as well as in animals treated with glucocorticoids [Kwai et al., 1985; Wang et al., 1977]. In humans, pharmacological doses of glucocorticoids – administered for anti-inflammatory and immunosuppressive therapies – have been reported to inhibit bone formation rates [Dempster, 1989] and increase the fatty marrow content of the proximal femur [Vande Berg et al., 1999]. These effects suggest that steroid treatment may inhibit osteoblastic differentiation by directing the osteoprogenitor cells along the adipocytic lineage.

Osteoblastic differentiation of fetal rat calvarial-derived [Owen et al., 1990; Pockwinse et al., 1992] and rat marrow stroma-derived [Kasugai et al., 1991; Malaval et al., 1994] progenitor cells has been characterized by temporal changes in cell morphology and gene expression. These culture models have demonstrated an ordered developmental sequence that can be defined in terms of three distinct stages: proliferation, extracellular matrix maturation, and matrix mineralization [reviewed by Lian and Stein, 1992]. Proliferation is marked by enhanced

expression of the cell-growth associated genes H4 histone and *c*-fos [Pockwinse et al., 1992]. During this period, genes associated with extracellular matrix formation (type I collagen, fibronectin, TGF-β) are expressed at peak levels. The rate of proliferation diminishes as cells form multilayered colonies (i.e., nodules). A gradient in cell morphology has been observed in these nodules, which consist of tightly-packed cuboidal cells in the center and spindle-shaped cells along the periphery [Malaval et al., 1994]. During the period of matrix maturation, the expression and activity of the bone/liver/kidney isoform of alkaline phosphatase increases to peak levels. Near the end of this developmental stage, nodule cells begin to mineralize the extracellular matrix. The expression of osteocalcin and bone sialoprotein increase with mineral deposition, while alkaline phosphatase levels begin to decline. Bone-like mineral deposition and the expression of mineral-related proteins can be used to identify a mature osteoblastic phenotype *in vitro*.

We hypothesize that glucocorticoid treatment *in vivo* will diminish the capacity of marrow stromal progenitor cells to differentiate into mature osteoblasts (i.e., their osteogenic potential) either by reducing the absolute number of osteoprogenitor cells or by making these cells less responsive to osteoinductive stimuli. As with marrow adipogenesis (described in section 2.3), this effect may be reversed following discontinuation of steroid treatment. To test this hypothesis, tibial marrow cells from methylprednisolone-treated rats were cultured in the presence of dexamethasone, β-glycerophosphate, and ascorbic-2-phosphate. After three weeks in culture, specific markers of the osteoblastic phenotype were measured, including the expression and secretion of osteocalcin and the formation of mineralized nodules. In addition, lipoprotein lipase expression was measured as a marker for adipogenic potential [Gimble, 1990]. Similar experimental models have been used to test for the effects of systemic administration of prostaglandin E_2 (PGE_2) [Keila et al., 2001; Weinreb et al., 1997] and skeletal unloading – either by tail-suspension [Kostenuik et al., 1997; Zhang et al., 1995] or sciatic neurectomy [Keila et al., 1994] – on rat marrow stromal cells.

3.2 Materials and Methods

3.2.1 *Animal protocols*

Juvenile female Spraque-Dawley rats were housed, treated, and sacrificed as described in section 2.2.1. The tibias from each animal were aseptically explanted and stored in 25 mL

Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 10% antibiotic/antimycotic (penicillin, streptomycin, neomycin, and fungizone; Gibco BRL).

3.2.2 *Marrow extraction from bones*

All cell culture work was performed in a laminar flow hood (NuAir, Plymoth, MN) using aseptic technique (following standard lab protocol). To reduce the risk of contamination, explanted tibias were immersed and swirled in 70% ethanol for approximately 15 seconds, then immersed in sterile phosphate buffered saline (PBS; Gibco BRL, Grand Island, NY) for at least one minute. The distal end of each tibia was trimmed at the proximal side of the intersection between the tibia and fibula. Bones were handled using sterile forceps and trimmed using sterile scissors. A 10 mL syringe (Becton Dickinson, Franklin Lakes, NJ) equipped with an 18-gauge needle (Becton Dickinson) was used to bore a hole into the soft marrow at the proximal end of the tibia. Sterile PBS was drawn into a second 10 mL syringe, also equipped with an 18-gauge needle. The tip of this needle was inserted into the hole bored by the first needle. The PBS was used to flush the marrow from the midshaft into a 100-mm culture dish (Fisher, Pittsburg, PA). The marrow from both tibias was flushed into a single culture dish, but marrow plugs from different animals were kept separate. (These plugs sometimes broke apart during the flushing process.)

3.2.3 *Marrow stromal cell culture*

The marrow from one tibia per animal was used to observe changes in osteoblastic potential of mesenchymal stem cells present in the marrow stromal population. As described above, the marrow plug was flushed from the tibia and dispersed into 10 mL of PBS. Large cell aggregates were broken up by repeatedly flushing the marrow suspension through 18 and 22-gauge needles. The cell suspension was transferred to a 50 mL centrifuge tube and centrifuged at 1000 rpm ($206 \times g$) for 5 minutes. The supernatant and floating fatty tissue were removed by vacuum aspiration. Special care was taken to avoid removing cells that had adhered to the conical section of the tube. The remaining cell pellet was re-suspended in 5 mL growth medium (DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic).

The concentration of nucleated cells in the suspension was counted using a hemacytometer (Hausser Scientific, Horsham, PA). Briefly, a 50 μL aliquot of the cell suspension was combined with 50 μL growth media and 100 μL 4% acetic acid (Fisher, Pittsburg, PA) to disrupt non-nucleated cells. The remaining cells were counted visually and converted to a concentration. Using this concentration an appropriate volume of suspension was added to each well of 12-well culture plates (Corning, Corning, NY) to achieve eight million nucleated cells per well. Sufficient growth media was added to bring the well volume up to 2 mL. The well plates were cultured in growth medium for one day to promote cell attachment to the tissue culture polystyrene. On the following day, growth medium was replaced with osteogenic medium (growth medium supplemented with 10 nM dexamethasone (Sigma, St. Louis, MO), 10 mM β-glycerophosphate (Sigma), and 37.5 μg/mL ascorbate-2-phosphate (Sigma)). The well plates were cultured in a 37°C, 5% CO_2, 95% relative humidity environment for 21 days. Osteogenic medium was replaced every 3 or 4 days.

3.2.4 *Secretion of osteocalcin*

After 21 days of culture, conditioned medium was collected to assay for osteocalcin secretion. Two days prior to sample collection, osteogenic medium was replaced with serum-free osteogenic medium supplemented with 1 nM 1,25-dihydroxyvitamin D_3. After 48 hours, medium from three wells – whose cells were derived from a single animal – were combined into a single 15 mL centrifuge tube (Fisher) and stored at –70°C until analysis. Levels of osteocalcin were measured using a rat osteocalcin Enzyme-Linked Immunosorbent Assay (ELISA) kit (Biomedical Technologies, Stoughton, MA) according to the manufacturer's instructions. Osteocalcin concentrations were determined using a set of standards (0.33 to 20 ng/mL) that were prepared from purified rat osteocalcin (provided in the kit). Briefly, samples were removed from storage at –70°C and thawed in a 37°C water bath for approximately 20 minutes. Three 100 μL aliquots of each sample were added to individual wells of a 96-well plate provided in the kit. Aliquots of the six standard solutions (100 μL/well) were also added in triplicate. Three aliquots of sample buffer (100 μL/well) were added to serve as blanks. After all samples were loaded, the well plate was covered with a transparent sealing tape and incubated at 4-8°C overnight. At the end of the incubation period, the well contents were removed and each well was rinsed three times with 300 μL PBS (provided with the kit). Next, a 100 μL volume of

osteocalcin antiserum was added to each well. The plate was covered with the sealing tape and incubated at 37°C for one hour. After the hour incubation, the antiserum solution was removed, the wells were rinsed three times with PBS, and a 100 μL aliquot of donkey anti-goat IgG peroxidase solution was added to each well. The plate was covered with the sealing tape and allowed to incubate at room temperature for one hour. After this incubation period, the donkey anti-goat IgG peroxidase solution was removed and the wells were rinsed three times with PBS. A solution of $3,3^1,5,5^1$-tetramethyl benzidine (TMB; a peroxidase substrate) was combined with hydrogen peroxidase solution and a 100 μL volume of this mixture was added to each well. The well plate was completely covered with aluminum foil and allowed to incubate at room temperature for 30 minutes. After the 30 minute incubation, 100 μL of stop solution (provided in the kit) was added to each well. The absorbance at 450 nm was then read with a FisherBiotech ELISA plate reader (Fisher). The average absorbance from the three blank wells was used for a background subtraction of the sample and standard measurements.

3.2.5 *RT-PCR analysis*

3.2.5.1 *RNA isolation from cell layers*

After removing the conditioned media from three wells to measure the levels of osteocalcin secretion at day 21, the cell layers were analyzed for expression of specific genes indicative of either osteoblastic or adipocytic differentiation. The three wells were washed twice with 2 mL sterile PBS per well. After the second aspiration step, a 250 μL aliquot of Lysis/binding solution – provided in a RNAqueous-4PCR kit (Ambion, Austin, TX) – was added to each of the wells. The wells were then vigorously scraped and the cellular material from all three wells was transferred to a single 1.5 mL microcentrifuge tube, yielding 750 μL of total sample per animal. The samples were stored at –70°C until analysis.

All solutions for RNA isolation were provided in the RNAqueous-4PCR kit and aliquots were used according to kit instructions. For analysis, the tubes were thawed in a 37°C water bath for approximately 20 minutes. The samples were vortexed and an equal volume of 64% (vol/vol) ethanol solution was added to the cellular material. For each sample, the total RNA was collected onto a fresh filter cartridge (provided with the kit) by vacuum filtration. Sequential volumes of two different wash solutions were used to rinse the RNA. The cartridge was then removed from vacuum and placed within a 1.5 mL collection tube (provided with the

kit). A 40 μL volume of 95-100°C elution solution was added to the center of each filter cartridge. The eluate (with dissolved RNA) was recovered by centrifugation at 14,000 rmp for ~30 seconds. An additional 20 μL volume of hot elution solution was added and the centrifugation was repeated. The filter cartridges were removed and the collection tubes were either stored at –70°C or immediately used for reverse transcription of total RNA within the eluate.

3.2.5.2 *Reverse transcription of total RNA to complementary DNA*

Reagents for reverse transcription were provided in a Superscript II First-Strand Synthesis for RT-PCR kit (Invitrogen, Carlsbad, CA) and used according to kit instructions. Frozen tubes of sample RNA were thawed at 37°C and gently mixed. A 20 μL aliquot from each sample was combined with 5 μL of random hexamers (50 ng/μL solution) and 2 μL of 10 mM dNTP mix in a 0.5 mL RNase-free microcentrifuge tube (Corning, Corning, NY). To denature the RNA, the RNA/primer mixtures were incubated at 70°C for 10 min in an Omnigene thermal cycler (Hybaid, Ashford, Middlesex, UK). The tubes were then placed on ice for a few minutes to allow random hexamer hybridization with the RNA strands. For each cDNA elongation reaction (one per sample), the following aliquots of reagents were added to the RNA/primer mixtures: 2 μL 10X RT buffer, 4 μL 25 mM $MgCl_2$, 2 μL 0.1 M DTT, and 1 μL RNaseOUT recombinant ribonuclease inhibitor. (The components were added in the order in which they are listed.) The solution was gently mixed and then briefly centrifuged. The tubes were incubated at 25°C for approximately two minutes. A 1 μL aliquot of SuperScript II reverse transcriptase was added to each tube and the completed reaction mixture was incubated at 25°C for 10 min to initially elongate the short cDNA strands (i.e., without their detachment from the RNA). The sample tubes were then incubated within the heating block of the thermal cycler at 42°C for 50 minutes. To terminate the reaction, the temperature was ramped once more to 70°C. The samples were then removed from the heating block and chilled on ice. A 1 μL aliquot of RNase H was added to each reaction tube and the tubes were incubated at 37°C for 20 min, degrading the total RNA present. The tubes were either stored at –20°C or immediately used for PCR.

3.2.5.3 *Polymerase chain reaction*

Primers were designed to specifically amplify complimentary DNA (cDNA) templates coding for osteocalcin and lipoprotein lipase mRNAs, as well as 18S ribosomal RNA (rRNA). The cDNA sequence for each protein was obtained from the Entrez nucleotide sequence database within the National Center for Biotechnology Information website [http://www.ncbi.nlm.nih.gov/Entrez/]. The primer pair sequences were formulated from these cDNA sequences using the PrimerSelect module within Lasergene (DNASTAR, Madison, WI). Each primer pair was selected based on the following factors: primer locations on the cDNA sequence, difference in melting temperature between the two primers, amplification product length, and optimal annealing temperature of the primers. The primers pairs used for target amplification are listed in Table 3.1.

Primer pair-specific mixtures (one per target sequence) were made before preparing the individual reaction mixtures for PCR (one per target per sample). All reagents for these mixtures were provided within a *Taq* PCR Master Mix kit (QIAGEN, Valencia, CA). A 38 μL volume of reagent mixture, required for each reaction, was composed of 16 μL DNase-free water, 1 μL of upper primer solution (100 μM; Ransom Hill Bioscience, Ramona, CA), 1 μL of lower primer solution (100 μM; Ransom Hill), and 20 μL *Taq* polymerase mix. The *Taq* polymerase mix is composed of 10X PCR buffer, 25 mM $MgCl_2$, 10 nM nucleotide triphosphate (NTP) mixture, and *Taq* DNA polymerase. For each sample, 38 μL of reagent mixture was combined with 4 μL of the sample cDNA mixture in a 0.5 mL microcentrifuge tube. For negative controls, 38 μL of reagent mixture was combined with 4 μL of DNAase-free water. Cloned product sequences for lipoprotein lipase, 18S rRNA, and β-actin were synthesized by Dr. William Huckle at the Virginia-Maryland Regional College of Veterinary Medicine. For positive controls, 4 μL of each product sequence was combined with 38 μL premix. A drop of light mineral oil (Fisher) was added to each tube to prevent evaporation and condensation of the reaction mixture during thermal cycling. The tubes were then gently mixed and briefly centrifuged.

The Omnigene thermal cycler was programmed to perform 30 amplification cycles as follows: cycle 1: 94°C for 2 min, 58.4°C for 1 min, 72°C for 2 min; cycles 2-29: 94°C for 1 min, 58.4°C for 1 min, 72°C for 1 min; cycle 30: 94°C for 1 min, 58.4°C for 1 min, 72°C for 10 min. Each cycle consisted of a denaturing step at 94°C, a hybridization step at 58.4°C, and an

elongation step at 72°C. At the end of the PCR cycles, the reaction tubes were removed from the heating block and cooled in a refrigerator (4-8°C) for at least 10 minutes.

3.2.5.4 *Gel electrophoresis and densitometry*

Agarose gels were prepared for size fractionation of the PCR product. A solution of 2% agarose (Gibco BRL, Grand Island, NY) in 1X Tris-Borate-EDTA (TBE) buffer (Gibco BRL) was prepared within an autoclaved 500 mL glass bottle (Wheaton). The agarose was melted in a microwave for two periods of approximately 60 seconds, with intermittent swirling, until it was homogenous. The bottle was placed under running water for about 30 seconds to expedite cooling. Approximately 100 mL of 2% agarose solution was immediately added to a gel casting tray (to a depth of ~8 mm). Two gel combs were positioned on the casting deck to mold 28 wells in the gel as it cooled. The casting tray was then cooled in a refrigerator for approximately 20 minutes. Additional gels were cast as needed from the hot agarose solution. Approximately 800 mL of 1X TBE buffer was added to a HORIZON horizontal gel electrophoresis device (Life Technologies, Gaithersburg, MD) so that its two electrodes were partially submerged. After sufficient cooling, the combs were carefully removed and the casting tray was completely immersed in the TBE bath with the wells positioned closer to the anode (black) lead.

The products in the PCR reaction volumes were separated according to size (i.e., base pair length) using gel electrophoresis. A 4 μL volume of 5X agarose gel loading buffer was added to each sample below the mineral oil layer. The reaction tubes were then gently mixed and briefly centrifuged. A 16 μL aliquot of each sample was loaded into individual wells of the gel. In order to determine the approximate size of products, l00-bp ladder solutions were also loaded onto the gel (typically added to the end wells of each gel.) For every 23 μL of ladder solution, 19 μL DNase-free water was combined with 2 μL of 100-bp ladder stock solution (New England BioLabs, Beverly, MA) and 2 μL 5X loading buffer. The electrophoresis device was connected to the terminals of a FisherBiotech FB600 voltage source (Fisher). The voltage source was set to 120 V and run for approximately 100 minutes. The 5X loading buffer was used to visually estimate the extent of band separation. After sufficient separation, the voltage source was deactivated and the gel was placed in a plastic dish containing 400 mL of 10^{-6} M ethidium bromide (Gibco BRL) in deionized water. The gel was gently swirled on a shaker (Fisher) for 15 minutes, transferred to another plastic dish containing with deionized water, and gently swirled

for an additional 15 minutes. The gel was then removed from the water bath, placed on a UV lamp (UVP, Upland, CA), and photographed using a Polaroid GelCam (Cambridge, MA).

The photograph was scanned using a ScanJet 3300C scanner (Hewlett Packard, Palo Alto, CA) and analyzed by densitometry using a custom subroutine (Appendix B-1) within ImagePro software (Media Cybernetics, Silver Spring, MD). In this subroutine, histograms were collected for a uniform area of interest (AOI) surrounding each band, and the histogram data was exported to Excel. The total intensity of each AOI was calculated in Excel by integrating the histogram. The intensity of a background AOI was used to adjust for noise. The mRNA band intensities for each sample were normalized by the 18S rRNA band intensity.

3.2.6 *Histochemical staining*

After 21 days of culture in 12-well plates, wells were assayed for mineralization of the extracellular matrix using the von Kossa stain as previously described (Goldstein, 2001). Osteogenic media was removed and the wells were rinsed twice with 2 mL sterile PBS. The cells were then fixed by adding 1 mL 10% neutral buffered formalin (Sigma) to each well. After a 10-minute incubation, the formalin was removed and the wells were rinsed twice with deionized water. A 0.5 mL volume of 5% $AgNO_3$ (Sigma) in water was added per well and the wells were exposed to incandescent light for approximately 30 minutes. The $AgNO_3$ solution was removed and the wells were washed twice more with deionized water. One drop of safrinin-O (Fisher) was added to each well, the plates were agitated for a period of approximately 30 seconds, and the wells were then rinsed three times with 100% ethanol to remove excess dye.

After allowing the wells to dry, images were acquired at 4X using an Olympus model IX50 inverted microscope (Opelco, Sterling, VA) equipped with a Prior motorized stage accessory and a Hamamatsu cooled CCD camera (I-cube, Crofton, MD). Images were tiled to produce a single image of an entire well (7028 x 7028 pixels) using an ImagePro subroutine (Appendix B-2). For each composite well image, a histogram of the individual pixel intensities – from 0 (black) to 4095 (white) – had a bimodal distribution. (The first peak corresponded to dark areas of mineralized matrix, while the second corresponded to cell membranes – stained with safrinin-O – and the well background.) The histogram data for each well image was exported to Excel. The minimum within the bimodal distribution – and the intensity corresponding to this minimum – was determined for each histogram. The average minimum

among all histograms was designated as the threshold for determining the total area of mineralization within each well. Image Pro was used to identify areas of mineralization greater than 300 μm^2 (i.e., the area of a cell ~20 μm in diameter) within the well perimeter (Figure 3.1). The total area of mineral coverage within each well was determined by summing the areas of the individual objects. For each animal, the total area of mineralization for the entire set of wells was normalized by the estimated number of nucleated marrow cells seeded into these wells.

3.2.7 *Statistics*

Each measurement is presented as the mean ± standard deviation for rats given a similar treatment regimen. Unless otherwise noted, one cumulative response was measured per stromal cell culture, and thus one response was measured per rat. A simple linear regression model was used to test for time-dependent trends over days 0–42 or days 0–70 (null hypothesis: slope = 0 for a linear model). A one-way analysis of variance in combination with Scheffe's multiple-comparison procedure was used to perform discrete pairwise comparisons of the means between treatment regimens. Statistical significance denotes a confidence of greater than 95% (α=0.05). Statistical analyses were performed using The SAS System for Windows release 8.02 (SAS Institute, Cary, NC), as described in Appendix A.

3.3 Results

3.3.1 Effect of methylprednisolone on the number of nucleated marrow cells

Osteoprogenitor cells were isolated by culture-adherence techniques from the non-adipocytic fraction of tibial marrow (i.e., the red marrow). Prior to culture, the number of nucleated red marrow cells per tibial explant was determined by counting these cells in suspension. The number of nucleated cells per tibia increased over the duration of this study for the untreated animals ($p<0.05$), as seen in Figure 3.2. However, the number of cells from the treated animals did not significantly increase with increasing duration of methylprednisolone treatment. Significantly fewer cells per tibia were counted for the day 14 and day 42 dosed animals versus sham controls ($p<0.05$). A significant increase in cell number was measured within the treated animals from day 42 to day 70 ($p<0.05$). Furthermore, no difference in cell number was found between treated and untreated animals at day 70.

3.3.2 Effect of methylprednisolone on osteocalcin secretion of cell cultures

Marrow stromal cells were seeded at eight million nucleated cells per well (~2.1 million cells/cm^2) onto 12-well culture plates. Cell cultures were maintained for 19 days in osteogenic media followed by 2 days in serum-free media supplemented with 1,25-dihydroxyvitamin D_3. Conditioned media was collected and analyzed for osteocalcin secretion – an indicator of osteoblast maturation – by the cell cultures. No differences were observed between the treated and untreated groups, nor were any systematic trends observed with duration of treatment (Figure 3.3).

3.3.3 Effect of methylprednisolone on the mRNA expression of cell cultures

RNA was isolated from cell layers after 19 days in osteogenic media followed by 2 days in serum-free media supplemented with 1,25-dihydroxyvitamin D_3. Total cellular RNA was probed for expression of osteocalcin mRNA, lipoprotein lipase mRNA, and 18S rRNA by RT-PCR. The PCR-amplified product sequences were separated by gel electrophoresis and quantified by densitometry of gel images. Individual product band intensities were then normalized by the band intensities for 18S rRNA (Figure 3.4). A slight time-dependent decrease in osteocalcin expression was measured for both treated and untreated animals (from days 0–70; $p<0.05$). However, no systematic trends in lipoprotein lipase expression were measured. No significant differences in expression between the treated and untreated groups were observed for either mRNA.

3.3.4 Effect of methylprednisolone on the deposition of bone-like mineral

Marrow stromal cells were cultured for 21 days and stained for mineralization. A tiled image was obtained for each well and an intensity threshold was used to identify regions of matrix mineralization (Figure 3.1). Regions of interest (>300 μm^2) were counted and their areas were summed to estimate the total coverage of mineral. Coverage areas were summed for the entire set of wells used for each animal, and the total was normalized by the number of nucleated cells seeded into the wells (Figure 3.5a). No significant difference between cultures obtained from treated and untreated animals was observed. In addition, no systematic trend in mineralization with respect to treatment duration was measured. A large variability in both the

number and size of nodules per well was observed, as represented by the well images in Figure 3.5b. Some of the wells did not yield any cell colonies.

3.4 Discussion

The central aim of this study was to evaluate the osteogenic potential of marrow stromal cells following their exposure to supraphysiological levels of glucocorticoid. Analysis of the bone marrow explants revealed a decrease in the number of non-adipocytic, nucleated marrow cells per tibia from rats treated with 18 mg/kg/day methylprednisolone for up to six weeks *in vivo*. However, cell numbers returned to near-control levels following cessation of treatment. When these marrow cells were cultured *in vitro* under osteogenic conditions, no significant differences in the levels of osteocalcin secretion or in the rates of bone-like mineral deposition were observed among the cultures. Furthermore, no differences in either osteocalcin or lipoprotein lipase messenger RNA expression were detected within cultures from steroid-treated rats versus controls. These messenger RNAs are indicators of mature osteoblasts and adipocytes, respectively [Beresford, 1984; Gimble, 1990]. The results indicate no detectable difference in the osteogenic potential of the marrow stromal cells following steroid treatment. However, supraphysiological steroid levels decrease the number of these cells *in vivo*.

Fewer nucleated marrow cells were harvested from the tibias of glucocorticoid-treated rats than from sham controls. Since buoyant adipocytes were removed from the marrow cell suspensions prior to culture, the decrease in nucleated cell number may correspond to a conversion of non-adipocytic (red) marrow into fatty (yellow) marrow. This explanation is consistent with the increase in fatty marrow volume observed in glucocorticoid treatment rats (described in section 2.3.4). The red marrow fraction is thought to contain the precursors for osteoblasts, adipocytes, hematopoiesis-supportive stromal cells, and other connective tissue phenotypes [Beresford, 1989; Caplan, 1991; Owen, 1988]. Glucocorticoid treatment may have directed adipogenesis of the progenitor cells within the marrow stroma while concurrently inhibiting other developmental pathways, altering the subpopulation make-up of the marrow. However, in addition to increasing fatty marrow volume steroid treatment also inhibited radial growth of the femora (described in section 2.3.2). Thus the steroid-induced decrease in nucleated marrow cells may be partially attributed to the reduction in bone size (i.e., in the total volume of the marrow compartment). After discontinuation of treatment, the number of

nucleated marrow cells and the volume fraction of fatty marrow both returned to near-control levels. Thus the effects of glucocorticoids on marrow composition appear to be reversible. Since the outer diameter of femurs from treated rats were still diminished after discontinuation of treatment, the glucocorticoid-induced reduction in red marrow cells may not solely be attributed to the decrease in bone size. Further speculation is difficult, however, due to the variability in marrow plug recovery.

The fibroblast-like precursor cells in the marrow stroma – which have varying degrees of lineage specificity – are thought to be derived from a subset of multipotent progenitor cells that may or may not be true stem cells (i.e., with the capacity for continuous self-renewal) [Satomura et al., 2000]. These multipotent progenitors supply the population of precursors through clonal expansion. Their direct progeny are directed along specific differentiation pathways by stimuli from the extracellular environment [Caplan, 1994]. A reciprocal relationship between the relative degrees of osteoblastic and adipocytic differentiation has been demonstrated within rat marrow stroma-derived cell cultures [Beresford et al., 1992]. The relationship between osteogenesis and adipogenesis may be important in the development of glucocorticoid-induced bone disease [Cui et al., 1997; Nuttall and Gimble, 2000]. Glucocorticoid administration has been reported to increase fatty marrow volume in human [Vande Berg et al., 1999] and rabbit [Kwai et al., 1985; Miyanishi et al., 2002; Wang et al., 1977] models of osteonecrosis. Using a cell line from the murine marrow stroma, Cui et al. [1997] reported that dexamethasone (a synthetic glucocorticoid) stimulated adipogenesis and concurrently suppressed osteogenesis in a dose-dependent manner. Based on these observations, glucocorticoid administration may diminish the osteogenic potential of osteoprogenitor cells within the marrow cavity (i.e., their number or responsiveness to osteogenic factors) by stimulating adipogenesis.

In order to assess the potential of marrow stromal cells to express markers of osteoblastic differentiation (i.e., their osteogenic potential), these cells were cultured in the presence of dexamethasone, β-glycerophosphate, and ascorbate-2-phosphate. Previous studies have demonstrated that these soluble factors induce osteoblastic differentiation within rat marrow stromal cell cultures [Kasugai et al., 1991; Malaval et al., 1994; Maniatopoulos et al., 1988; Peter et al., 1998]. An osteoblast-like cell phenotype has been characterized *in vitro* by the expression of the bone-specific proteins osteocalcin and bone sialoprotein, as well as the formation of mineral deposits that are analogous to hydroxyapatite [Malaval et al., 1994; Maniatopoulos et al.,

1988]. For this study, markers of osteoblastic differentiation (osteocalcin expression, osteocalcin secretion, and mineral deposition) were observed in all of the cultures. However, no significant differences in the level of these markers were measured between cell cultures from treated and control rats. The results suggest that methylprednisolone administration did not alter the osteogenic potential of marrow stromal cells.

As described in section 3.3, there were large variances in the measured responses from the cell cultures. These variances may be attributed to the heterogeneous nature of primary marrow stromal cell cultures, which initially contain both hematopoietic and mesenchymal cell phenotypes. Previous studies have reported that osteoprogenitor cells make up about $1/10^5$ of the total nucleated marrow cells seeded *in vitro* [Aubin, 1999; Rickard et al., 1996]. In the current study, a distribution in the size of bone nodules was observed (Figure 3.5b). Relatively large nodules may have been formed from multiple osteoprogenitor cells in close proximity following initial seeding. It appears that matrix mineralization, and possibly other osteogenic markers, were most prevalent in relatively large bone nodules. This observation is consistent with previous studies that have demonstrated enhanced mineralization associated with increased osteoprogenitor seeding density [Aubin, 1999, Herbertson and Aubin, 1995; Jaiswal et al., 1997]. In a more recent study, rat osteoprogenitor cells were seeded as dense clusters in secondary culture, resulting in large nodules that were heavily mineralized [Goldstein, 2001]. These results support the hypothesis that cell-cell interactions are important in osteoblastic differentiation [Lecanda et al., 1998; Cheng et al., 1998]. Therefore, the large variations seen in the results may be a function of the variances in osteoprogenitor cell distribution among the cultures. Alternatively, these variations might be caused by rat-to-rat variations in bone marrow properties. Recent studies have reported donor-dependent variations in the osteogenic potential of human marrow stromal cells *in vitro* [Phinney et al., 1999; Walsh et al., 2001].

The results presented in this study suggest that glucocorticoid administration does not affect the osteogenic potential of uncommitted progenitor cells from the marrow stroma. Although significantly fewer nucleated cells were harvested from rats that were continuously treated with glucocorticoid for 6 weeks, these cell numbers rebounded following discontinuation of treatment. A similar trend was observed regarding the volume of fatty marrow, indicating that a steroid-induced conversion of red marrow into yellow marrow is reversible. *Ex vivo* culture of marrow stromal cells demonstrated that glucocorticoids did not affect either the number of

marrow progenitor cells or their responsiveness to osteogenic stimuli. Instead, glucocorticoid administration may suppress the osteoblastic differentiation of their clonogenic progeny in favor of adipogenesis. The resulting decrease in osteoblast recruitment may contribute to the inhibition of bone formation that has been attributed to steroid treatment [Dempster, 1989; Weinstein et al., 1998a].

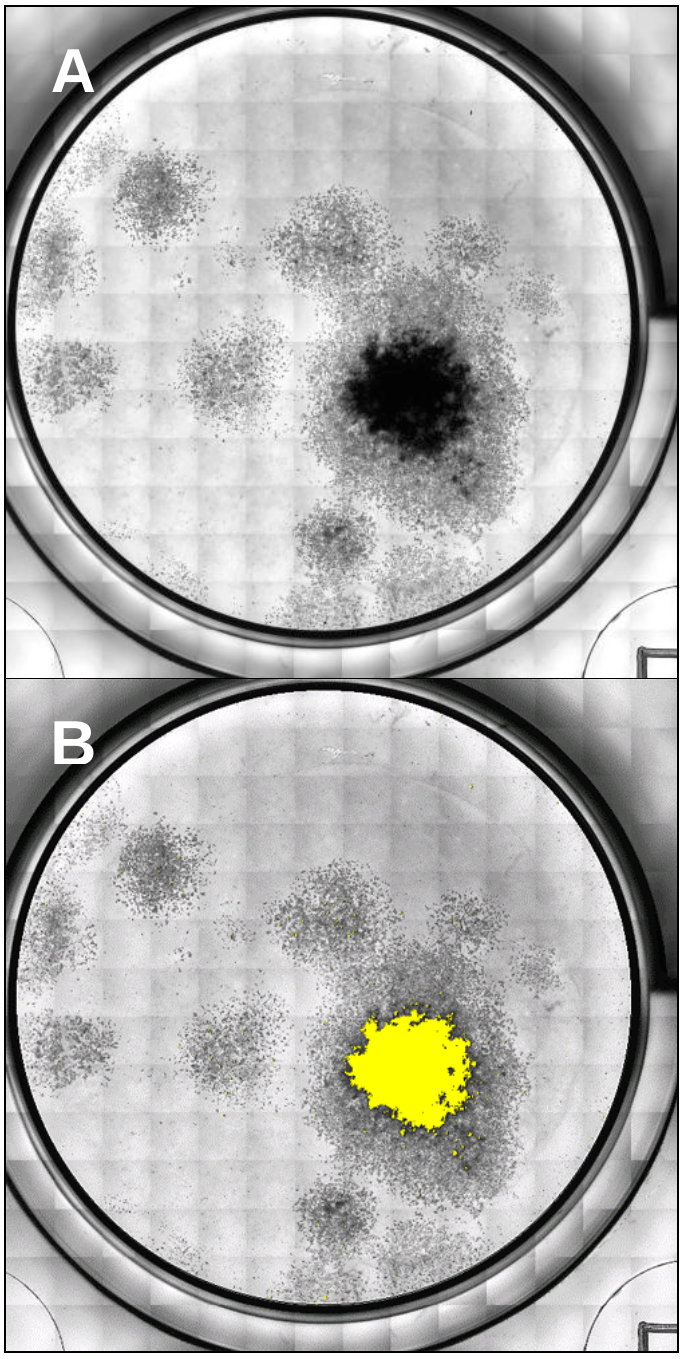

Figure 3.1: Analysis of mineral deposition by histochemical staining. (a) The tiled image (at 4X magnification) of a typical well culture is presented. Cell clusters appear dark gray, while regions of mineral deposition appear black. The histogram of individual pixel intensities – ranging from 0 (black) to 4095 (white) – has a binodal distribution, in which the first peak corresponds to the areas of mineral deposition. (b) Regions of mineralization greater than 300 μm^2 (highlighted in yellow) were used to determined the total area of mineralization. The well diameter is approximately 22 mm.

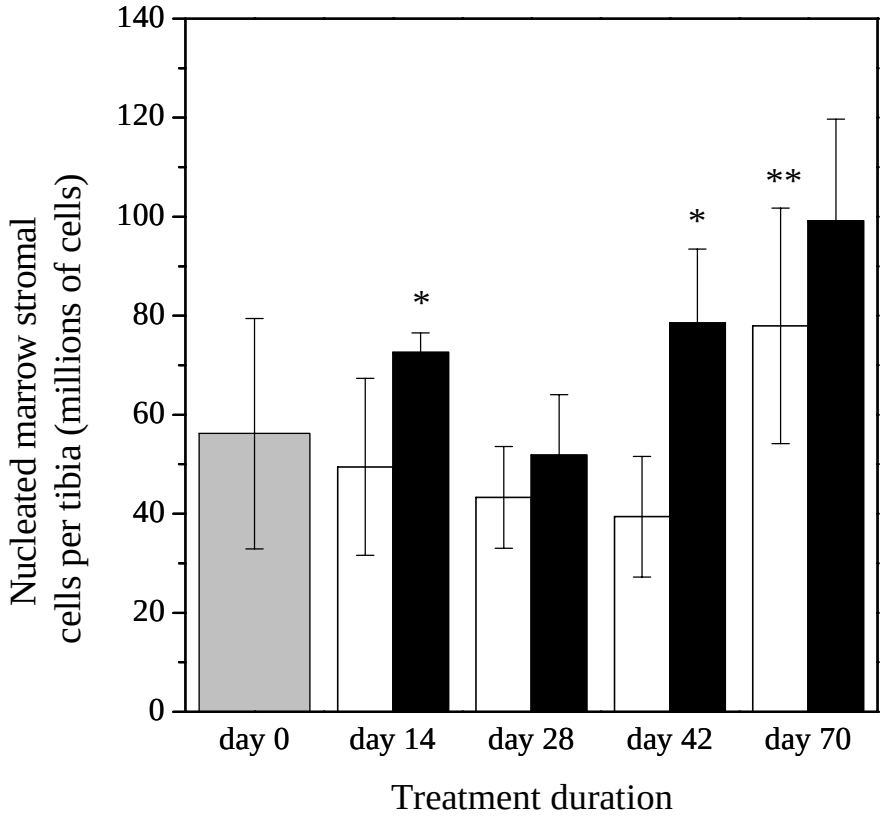

Figure 3.2: Nucleated cell per tibia. The number of nucleated marrow cells obtained from a single tibia (not including buoyant adipocytes) is plotted as a function of treatment duration. Rats were given either methylprednilosone (white) or sham (black) treatment from day 0 (gray) to day 42. Error bars correspond to the standard deviation of n = 4 (n = 6 for white bar, day 70) tibias. A single asterisk (*) denotes a statistically significant difference in cell numbers between treated and untreated rats (at a single timepoint). A double asterisk (**) denotes a statistically significant increase in cell number from day 42 to day 70 (i.e., the 4-week recovery period).

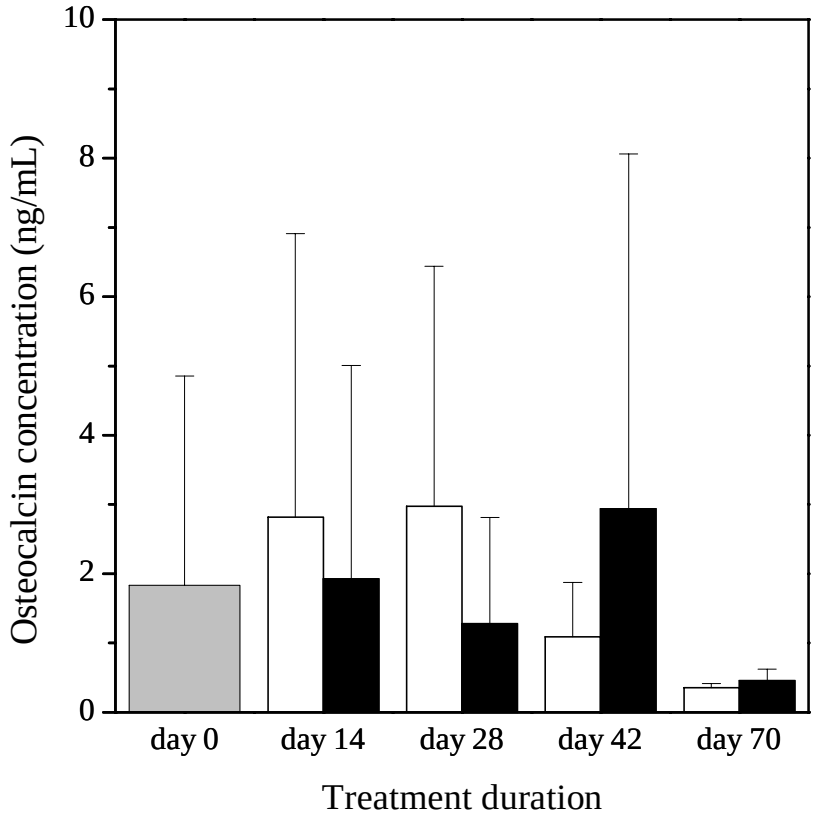

Figure 3.3: Osteocalcin secretion after 21 days in culture. Osteocalcin concentration in the conditioned media of cell cultures derived from treated (white) and untreated (black) animals are plotted as a function of treatment duration. Cultures were maintained in growth medium for one day, followed by 19 days in osteogenic medium, and then 2 days in serum-free medium supplemented with 1,25-dihydroxyvitamin D_3. Error bars correspond to the standard deviation of n = 4 (n = 6 for day 70, treated) animals (3 wells per animal). The large error bars reflect undetectible osteocalcin in one or more samples, which skewed the mean.

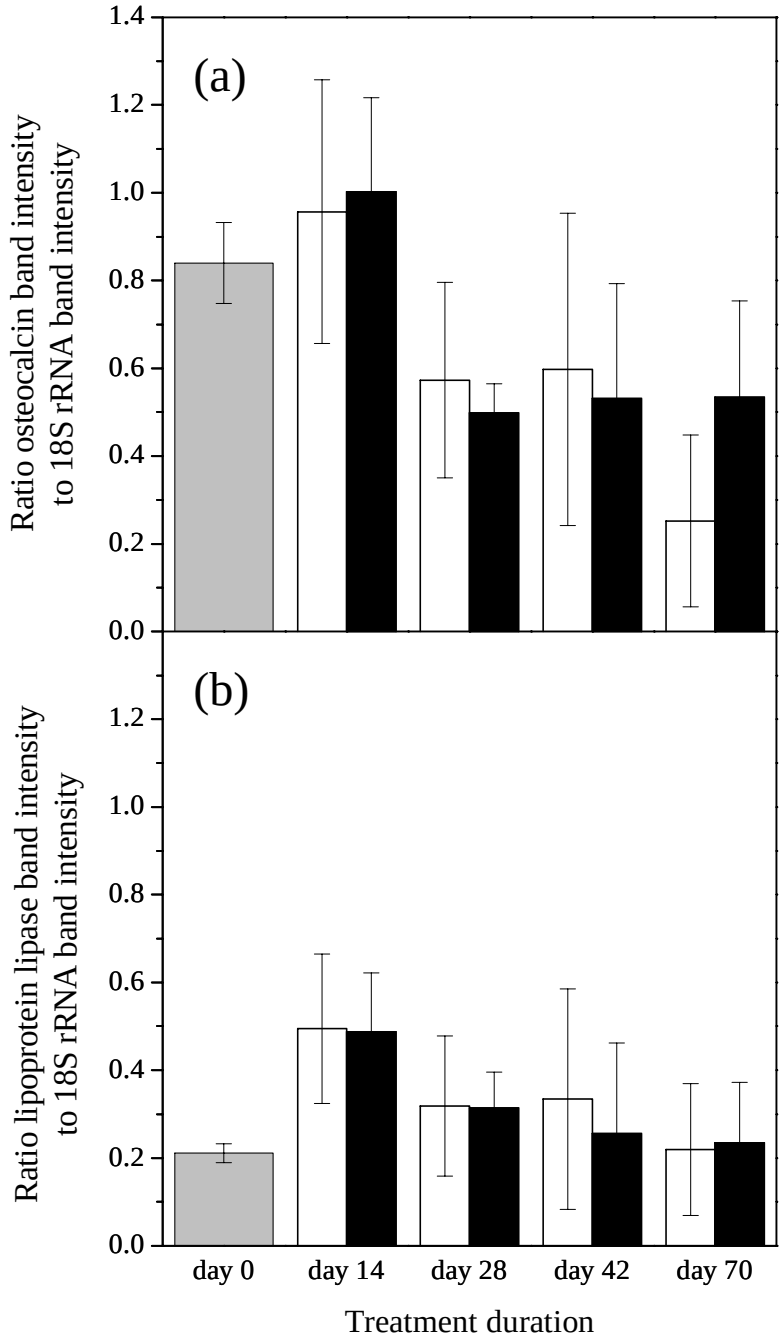

Figure 3.4: Gene expression after 21 days in culture. Relative levels of (a) osteocalcin and (b) lipoprotein lipase mRNA to 18S ribosomal RNA expressed by marrow stromal cell cultures obtained from treated (white) and untreated (black) animals are plotted as a function of treatment duration. Cultures were maintained in growth media for one day, followed by 19 days in osteogenic media, and then 2 days in serum-free media supplemented with 1,25-dihydroxyvitamin D_3. Error bars correspond to the standard deviation of n = 4 (n = 6 for day 70, treated) animals (3 wells per animal).

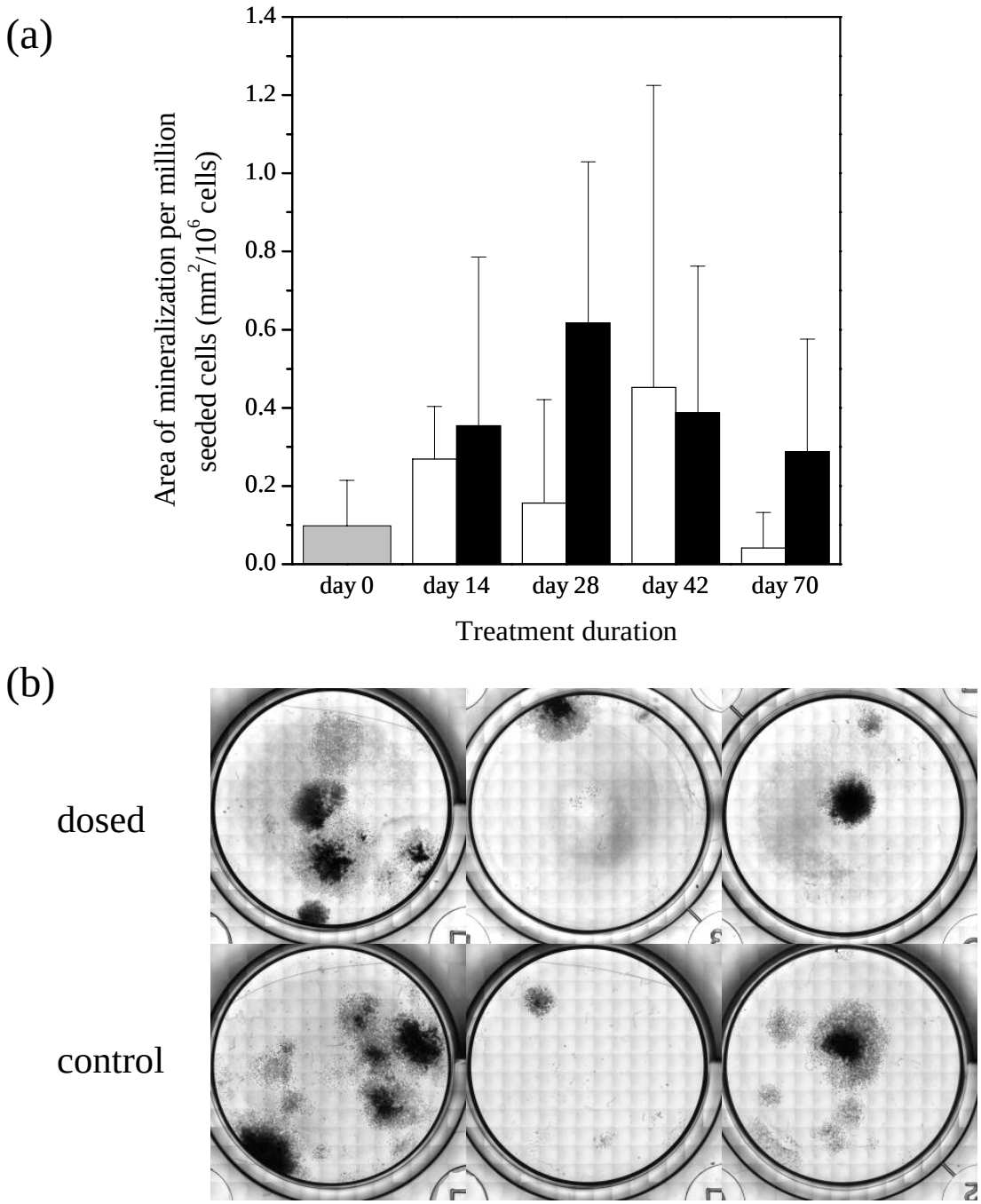

Figure 3.5: Deposition of mineral after 21 days in culture. (a) The total area of bone-like mineral deposits in cell cultures derived from treated (white) and untreated (black) animals are normalized by the total number of seeded cells and plotted as a function of treatment duration. Cultures were maintained in growth medium for one day, followed by 21 days in osteogenic medium. Error bars correspond to the standard deviation of n = 4 (n = 6 for day 70, treated) animals. (b) Three typical well images from the cultures of dosed and control rats are presented to demonstrate the variability in the formation of cell colonies. The well diameters are all approximately 22 mm.

Target	Primer	Sequence
Osteocalcin mRNA	sense	5’-AGACTCCGGCGCTACCTCAA-3’
	antisense	5’-CAGCTGTGCCGTCCATACTTTC-3’
Lipoprotein lipase mRNA	sense	5’-GTATCGGGCCCAGCAACATTATCC-3’
	antisense	5’-GCCTTGCTGGGGTTTTCTTCATTC-3’
18S rRNA	sense	5’-CGGCGCCCCCTCGATGCTCTTA-3’
	antisense	5’-TCCTGGTGGTGCCCTTCCGTCAAT-3’

Table 3.1: Primer pairs for the amplification of complementary DNA templates for osteocalcin mRNA, lipoprotein lipase mRNA, and 18S rRNA by RT-PCR. Primers were designed using the PrimerSelect module within Lasergene.

Chapter 4:

Examination of dexamethasone treatment duration on osteoblastic differentiation of bone marrow stroma in vitro

4.1 Introduction

Currently, autogenous cancellous and cortical bone grafts are most commonly used for the repair of clinically significant bone defects [Stevenson, 1998]. However, donor-site morbidity is an important concern [Younger and Chapman, 1989]. A promising alternative for the repair of critically-sized defects (i.e., requiring surgical intervention) involves culturing autologous osteoprogenitor cells within porous biodegradable scaffolds under conditions that stimulate osteoinduction [Goldstein et al., 2001]. Osteoprogenitor cells can be isolated from the bone marrow stroma, expanded extensively in culture without significant lineage progression, and then stimulated to develop into bone-like tissue *in vitro* [Bruder et al., 1997; Haynesworth et al., 1992; Jaiswal et al., 1997] and *in vivo* [reviewed by Bruder et al., 1998]. The advantage of this strategy is that a small amount of autologous marrow aspirate is required for bone regeneration. To expedite the healing process, it may be advantageous to initiate progenitor cell differentiation along the osteoblastic lineage prior to implantation of the scaffold [Bruder et al., 1998]. However, progenitor cells from the marrow stroma have multilineage potential *in vitro*: depending on the culture conditions, these cells have been shown to differentiate into osteoblasts [Haynesworth et al., 1992; Maniatopolous et al., 1988], adipocytes [Beresford et al., 1992; Dennis and Caplan, 1996], chondrocytes [Johnstone et al., 1998], and other connective tissue phenotypes. To specifically induce osteoblastic differentiation, osteogenic factors must be introduced in the appropriate quantities and sequence.

Osteoblastic differentiation *in vitro* is marked by three distinct stages of cellular activity: proliferation, extracellular matrix maturation, and matrix mineralization [reviewed by Lian and Stein, 1992]. Initially, osteoprogenitor cells (i.e., undifferentiated fibroblast-like cells derived from putative mesenchymal stem cells) are highly mitotic, as demonstrated by the expression of the cell-growth associated genes H4 histone and *c*-fos [Pockwinse et al., 1992]. During this proliferation stage, genes associated with extracellular matrix formation (type I collagen, fibronectin, TGF-β) are also expressed at peak levels. As cells form multilayered colonies (i.e., nodules), the rate of proliferation decreases and the expression of the bone/liver/kidney isoform

of alkaline phosphatase increases to peak levels. Following the period of matrix maturation, nodule cells begin to mineralize the extracellular matrix. The expression of osteocalcin and bone sialoprotein increases with mineral deposition, while alkaline phosphatase levels decline.

Dexamethasone, a synthetic glucocorticoid, has been demonstrated to be an effective but nonspecific inductor of osteoblastic differentiation [Beresford et al., 1992; Cheng et al., 1994; Cui et al., 1997; Maniatopoulos et al., 1988]. The effects of dexamethasone on progenitor cell proliferation and phenotypic development have been reported to depend on both the dosage and timing of treatment [Atmani at el., 2002; Bellows et al., 1987; Beresford et al., 1992]. While necessary for directing osteoblastic differentiation, dexamethasone also stimulates adipocyte formation in both primary marrow stromal cell cultures [Bennett et al., 1991; Beresford et al., 1992] and marrow stroma-derived cell lines [Cui et al., 1997]. Furthermore, dexamethasone has been shown to inhibit the expression of type I collagen, the primary structural protein of bone matrix, as well as the proliferation of osteoprogenitor cells in both human and rat marrow stromal cell cultures [Cheng et al., 1994; Kim et al., 1999; Leboy et al., 1991]. Thus we hypothesize that removing the glucocorticoid after initial osteoinduction will enhance bone-like tissue formation *in vitro*.

In this study, osteoprogenitor cells – isolated from rat bone marrow and expanded in primary culture – were induced to differentiate in the presence of the following osteogenic supplements: dexamethasone, β-glycerophosphate, and ascorbate-2-phosphate. To assess the effects of dexamethasone on the progression of osteogenesis, the glucocorticoid was removed from the culture medium after 3, 7, 10, 17, 24, or 31 days in secondary culture. Cell number (a marker of proliferation) and collagen synthesis were measured at days 10 and 14. The expression of messenger RNAs for osteoblastic (osteocalcin, bone sialoprotein) and adipocytic (lipoprotein lipase) markers were analyzed at day 24, while areas of bone-like mineral deposition were quantified at day 31.

4.2 Materials and Methods

4.2.1 *Animal protocols*

Juvenile male Spraque-Dawley rats (125-150 g; Harlan, Dublin, VA) were housed and sacrificed at the Lab Animal Resources (LAR) facility using university-approved protocols. The rats were housed in a 25°C, 12-hr light / 12-hr dark environment two days prior to sacrifice; they

were provided with Purina rat chow and water *ad libitum*. The rats were sacrificed by CO_2 inhalation in the necropsy room of the LAR facility. Aseptic extraction of the hind legs was performed within an operating room at this facility. Briefly, the rats' hind quarters were shaved with clippers and sterilized using sequential washings with betadine and ethanol solutions. Each hind leg was exarticulated at the hip joint, the femur and tibia were separated from the knee joint, and attached musculature was removed by gentle scraping. Tibias and femurs were placed in 50 mL centrifuge tubes (Fisher, Pittsburg, PA) containing 25 mL Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 10% antibiotic/antimycotic (penicillin, streptomycin, neomycin, and fungizone; Gibco BRL). The tubes were then transported to the tissue culture facility for marrow plug extraction from bones. Animal carcasses were disposed of according to LAR procedures.

4.2.2 *Marrow stromal cell primary culture*

All cell culture work was performed in a laminar flow hood (NuAir, Plymoth, MN) using aseptic technique. The proximal epiphysis of each femur or the distal epiphysis of each tibia (at the proximal side of the intersection between the tibia and fibula) was trimmed prior to marrow extraction. A 10 mL syringe (Becton Dickinson, Franklin Lakes, NJ) equipped with an 18-gauge needle (Becton Dickinson) was used to bore a hole into the soft marrow at the proximal end of the tibia or distal end of the femur (end adjacent to the knee joint). Sterile growth medium (DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic) was drawn into a second 10 mL syringe, also equipped with an 18-gauge needle. The tip of this second needle was inserted into the hole bored by the first. The 10 mL medium was used to flush the marrow from the midshaft of all six bones into the same 100-mm culture dish (Fisher). Marrow plugs from each 50 mL tube were flushed into separate culture dishes. Large cell aggregates were broken up by repeatedly flushing the marrow suspensions through 18 and 22-gauge needles. The dispersed cell suspensions were transferred to 50 mL centrifuge tubes and pelleted by centrifugation at 1000 rpm ($206 \times g$) for 5 minutes. The supernatant and floating fatty tissue were removed by vacuum aspiration. Special care was taken to avoid removing cells that had adhered to the conical section of the tube. Each pellet was re-suspended in 6 mL growth medium. The cells from the two suspensions were then distributed into six 100-mm culture

dishes (2 mL/dish). Sufficient growth medium was added to bring each dish volume up to 7 mL. The cells were cultured in a 37°C, 5% CO_2, 95% relative humidity environment for 14 days. Growth medium was replaced every 3 or 4 days.

4.2.3 *Dexamethasone treatment study (secondary culture)*

Following 14 days in primary culture, marrow stromal cells were subcultured in the presence of the osteogenic supplements dexamethasone, β-glycerophosphate, and an ascorbate analog. Briefly, growth medium was removed and each dish was washed twice with 5 mL phosphate-buffered saline solution (PBS; Gibco BRL). To lift the adherent cells, 2 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco BRL) was added to each dish and the dishes were incubated at 37 °C for 10 minutes. Six mL of growth medium was added to each dish to dilute the trypsin. Any cells still attached to the dish surface were removed by repeatedly rinsing the surface with the diluted cell suspension. The cell suspensions were then collected into two 50 mL tubes and centrifuged at 1000 rpm ($206 \times g$) for 5 minutes. Each pellet was resuspended in 5 mL medium and the concentration of suspension was estimated with a hemacytometer. Based on these concentrations, an appropriate volume of suspension was added to each well of 12-well culture plates (Corning, Corning, NY) to achieve a seeding density of 1×10^5 cells/well (2.6×10^4 cells/cm^2, or ~50 of confluence). Sufficient growth medium was then added to bring each well volume up to 2 mL. Cells were allowed to attach overnight in a 37°C, 5% CO_2, 95% relative humidity environment. The following day (hereafter designated as day 0), growth medium from each 12-well plate was replaced with 'osteogenic' medium (growth medium supplemented with 10 nM dexamethasone (Sigma, St. Louis, MO), 10 mM β-glycerophosphate (Sigma), and 37.5 μg/mL ascorbate-2-phosphate (Sigma)).

Well plates were assayed at the following timepoints after initial dexamethasone administration: days 10 and 14 for measurements of cell number and collagenous protein synthesis; day 24 for Northern blot analysis; day 31 for histochemical staining of mineral deposition. Medium changes were made at days 3, 7, 10, 14, 17, 21, 24, 27, and 31. Osteogenic medium was replaced with medium lacking dexamethasone (but still containing β-glycerol phosphate and ascorbate-2-phosphate) at the following timepoints: days 3 or 10 for wells assayed at days 10 and 14; days 10 or 17 for wells assayed at day 24; days 3, 7, 10, 17, or 24 for wells

assayed at day 31. For each assay timepoint, wells with continuous dexamethasone supplementation were used as controls.

4.2.4 *Measurement of cell number*

Total cell number within each well was ascertained by flourometric analysis of DNA content [Goldstein et al., 2001; Ishaug et al., 1997; West et al., 1985]. Osteogenic medium was removed and the wells were rinsed twice with two mL sterile PBS. A 200 μL aliquot of 10 mM EDTA (pH 12.3) (Sigma) was added to each of the wells. The wells were then vigorously scraped and an additional 500 μL of 10 mM EDTA was added to each well. The aliquot was transferred to a single 1.5 mL microcentrifuge tube, and an additional 700 μL was added to remove any remaining material. The 1.4 mL samples were stored at –70°C until analysis.

Seven DNA standards (0, 1, 2, 3, 4, 6, and 8 μg DNA) were prepared by diluting various aliquots (0 to 160 μL) of a 50 μg/mL DNA stock solution to a total volume of 1.4 mL with 10 mM EDTA. The concentration of DNA stock was verified by absorbance at 260 nm (50 μg/mL DNA has an absorbance of 1.00 at this wavelength). Samples were thawed and sonicated on ice for 10 minutes. To neutralize the pH, 200 μL of 1 M KH_2PO_4 was added to each sample. The standards were sonicated on ice for 10 minutes and 200 μL of 1 M KH_2PO_4 was added to each tube. Samples and standards were thoroughly mixed prior to analysis.

For analysis, a 500 μL aliquot of each sample or standard was combined with 1.5 mL of 100 ng/mL Hoechst 33258 dye (Sigma) in 100 mM NaCl (Sigma) and 10 mM Tris-HCl (pH 7.0) (Sigma) within a quartz cuvette. Fluorescent measurements were collected in duplicate with a DyNAQuant 200 (Hoefer, San Franscisco, CA). A standard curve was constructed to correlate flourescence and mass of DNA. A conversion factor of 10.4 pg rat DNA/cell [Goldstein et al., 2001] was used to calculate cell number for each sample.

4.2.5 *Collagen synthesis*

Collagen synthesis was estimated by the incorporation of [^{3}H]-proline as previously described [Goldstein, 2001; Ishaug et al., 1994]. Proline is present at a relatively high frequency in collagenous protein [Puleo et al., 1991]. Twenty-four hours prior to sample collection, 6 μL of 1 μCi/μL L[5-^{3}H]-proline stock solution (ICN, Irvine, CA) was added to the culture medium

to a final concentration of 3 μCi/mL. After the 24-hour incubation, the wells were rinsed twice with 2 mL PBS. A 1 mL volume of nonspecific digestion solution (1 mg/mL pepsin (Sigma) in 1 M acetic acid (Fisher)) was added to each well, after which the wells were incubated for 4 hours at room temperature. The wells were rigorously scraped and two 200 μL aliquots were transferred to individual 1.5 mL microcentrifuge tubes. For each sample, 100 μL of 0.6 M *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulfonic acid (HEPES, pH 7.0; Sigma) and 200 μL of a collagen-specific digestion solution (6.25 mM n-ethyl-maleimide (Sigma), 1.25 mM $CaCl_2 \cdot 2H_2O$ (Sigma) and 125 μg/mL collagenase (Sigma #C0773) in 0.02 N HCl (Fisher)) were added to the tube. The tubes were incubated in a 37°C water bath for 90 minutes. The digestion was then quenched by adding a 500 μL aliquot of 10 wt% trichloroacetic acid and 0.5 wt% tannic acid (10% TCA/0.5% TA). The tubes were mixed, chilled on ice for approximately 10 minutes, and centrifuged at 2,000 x g for 5 minutes. A 900 μL aliquot of each supernatant was added to individual 5 mL scintillation vials. The pellets were resuspended in 500 μL of 5% TCA/0.25 %TA solution, chilled on ice, and centrifuged again at 2000 x g for 5 minutes. Each 500 μL aliquot of this supernatant was added to the same scintillation vials as before, yielding a total of 1.4 mL supernatant per subsample. These vials were designated as containing the collagenase-digestible protein fraction. The pellets were resuspended with 1.3 mL of 5% TCA/ 0.25% TA, and the entire tube volumes were transferred to separate scintillation vials. These vials were designated as containing the non-collagenase-digestible protein fraction. A 4 mL volume of immulsion type liquid scintillant was added to each vial, and counts were measured using a Tricarb 2100TR (Packard) beta-counter. Counts for collagenase-digestible and non-digestible fractions were normalized using the calculated mean cell densities. The percent collagenase protein (CP) for each sample was calculated using the following formula [Ishaug et al., 1994]:

$$\%\,\mathrm{CP} = \frac{\mathrm{CP}}{\left(\mathrm{CP} + 5.2 \times \mathrm{NCP}\right)}$$

4.2.6 *Northern blot analysis*

4.2.6.1 *RNA isolation from cell layers*

Cell layers were analyzed for expression of phenotypic markers of either osteoblastic or adipocytic differentiation. Cells were supplemented with dexamethasone for either 10, 17, or 24 days. On the twenty-fourth day, wells were washed twice with 2 mL sterile PBS per well. Total

cellular RNA was isolated using solutions provided in a RNAqueous-4PCR kit (Ambion, Austin, TX). Briefly, 500 μL of lysis/binding solution was added to each of the wells. The wells were vigorously scraped and the cellular material was transferred to individual 1.5 mL microcentrifuge tubes. Samples were stored at –70°C until analysis. For analysis, the tubes were thawed in a 37°C water bath for approximately 20 minutes. The samples were then vortexed and an equal volume of 64% (vol/vol) ethanol solution was added to the cellular material. For each sample, the total RNA was collected onto a fresh filter cartridge (provided with the kit) by vacuum filtration. Sequential volumes of two different wash solutions were used to purify the RNA. The cartridge was removed from vacuum and placed within a 1.5 mL collection tube (provided with the kit). A 60 μL volume of 95-100°C elution solution was added to the center of each filter cartridge. The eluate was then recovered by centrifugation at 14,000 rmp for ~30 seconds. An additional 60 μL volume of hot elution solution was added and the centrifugation was repeated. The filter cartridges were removed and the concentration of RNA within each eluate was determined by combining 10 μL of sample with 990 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, adjusted to pH 8.0) and measuring the absorbance at a wavelength of 260 nm. This absorbance was multiplied by the dilution factor (100) and a conversion factor of 40 μg/mL RNA per unit absorbance. The samples were stored at –70°C until use.

4.2.6.2 *RNA fractionation and transfer to membranes*

Unless otherwise noted, reagents for Northern blot analysis were provided in a NorthernMax kit (Ambion) and used according to kit instructions. Total RNA samples were thawed and 12 μg of RNA from each sample was lyophilized and reconstituted with 18 μL formaldehyde load dye and 2 μL of 100 μg/mL ethidium bromide (Gibco BRL) solution. A denaturing 1% agarose gel was prepared for RNA fractionation by electrophoresis. For each gel, 1 g agarose was added to 90 mL diethyl pyrocarbonate (DEPC; ICN, Costa Mesa, CA)-treated water and melted in a microwave until the solution was completely homogenous. The solution was then cooled in a 60°C water bath for approximately 15 min. Following thermal equilibration, 10 mL of 10X denaturing gel buffer was added to the agarose solution, and the solution was immediately poured into a gel casting tray to a thickness of 6 mm. Prior to pouring, a 14-tooth gel comb was positioned on the casting tray to form wells for sample loading. While allowing the gel to solidify, 80 mL of 10X MOPS gel running buffer was diluted to 800 mL with

DEPC-treated water. The running buffer was added to a HORIZON horizontal gel electrophoresis device (Life Technologies, Gaithersburg, MD). After the gel had solidified, the comb was removed and the casting tray was immersed in the running buffer with the wells positioned closer to the anode (black) lead. The reconstituted RNA samples were incubated in a heat block at 65°C for 15 min and subsequently loaded into adjacent wells of the gel. The electrophoresis device was connected to the terminals of a FisherBiotech FB600 voltage source (Fisher). Electrophoresis was conducted at 100 V until the bromophenol blue dye (corresponding to 500-nucleotide RNA) had migrated about ¾ of the gel length (approximately 90 min). The gel was removed from the running buffer, wrapped in plastic wrap, placed on a UV lamp (UVP, Upland, CA), and photographed using a Polaroid GelCam (Cambridge, MA) to confirm RNA fractionation.

RNA from the gel was transferred overnight to the surface of a Zeta-Probe GT Genomic Tested Blotting Membrane (BioRad, Hercules, CA) by capillary transport. Briefly, a glass baking dish was filled with 500 mL of 20X saline-sodium citrate (SSC) transfer buffer (3 M sodium chloride (Sigma), 0.3 M sodium citrate (Sigma)). A gel casting tray was inverted and placed in the bath, forming an island within the buffer. A piece of Whatman 3MM chromatography paper (Whatman, Maidstone, England) was cut to the same width as the casting tray, but about three times the length. This piece was made into a wick by wrapping the ends of the paper around the casting tray and submerging them in the transfer buffer. The remainder of the transfer apparatus was set up as follows (from bottom to top): 3 pieces of chromatography paper (cut to the same dimensions as the casting tray, pre-wet in transfer buffer); the agarose gel; 4 strips of Parafilm placed along the gel edges (to prevent capillary transport around the ends of the gel); one Zeta-Probe membrane (cut to dimensions slightly greater than the gel area left exposed by the Parafilm strips, pre-wet in transfer buffer); 2 additional pieces of chromatography paper (dry); a stack of paper towels (~5 cm thick); a weight (~175 g) to compress the stack. Following overnight transfer, the membrane was briefly rinsed (~10 sec) in gel running buffer and wrapped in plastic wrap. The RNA was then crosslinked to the membrane using a Stratalinker 1800 UV Crosslinker (Stratagene, La Jolla, CA) operated in the autocrosslink mode. The wet membrane was stored at –20°C in a 50 mL centrifuge tube.

4.2.6.3 *Probe hybridization and detection*

Complimentary DNA (cDNA) probes to the RNAs for osteocalcin, lipoprotein lipase, bone sialoprotein, and 18S ribosomal RNA were prepared by Dr. William Huckle (Virginia-Maryland Regional College of Veterinary Medicine). Probes were randomly labeled with [α-^{32}P]-dATP using a Prime-a-Gene kit (Promega, Madison, WI) according to kit instructions. Unless otherwise noted, all reagents for labeling were provided in this kit. Briefly, 25 ng of a probe solution (~10 ng/μL) was diluted to a volume of 30 μL using nuclease-free water. The probe was then denatured at 95°C for 3 min using a heat block, then rapidly chilled on ice (~2 min). The following components were added in the order in which they are listed: 10 μL of 5X labeling buffer; 2 μL of an equimolar mixture of CTP, GTP, and TTP; 2 μL of nuclease-free bovine serum albumin (BSA); 5 μL of [α-^{32}P]-dATP (50 μCi, 3,000Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, England); 1 μL DNA polymerase I (Klenow) fragment). The mixture was gently mixed and incubated at room temperature for one hour. To terminate the labeling reaction the mixture was heated at 95°C for 3 min and chilled on ice for 2 min. A 5 μL aliquot of 0.2 M EDTA (pH 8.0) was then added to the mixture.

Unless otherwise noted, reagents for probe hybridization were provided in the NorthernMax kit. ULTRAhyb hybridization buffer was preheated to 68°C in a water bath. Membranes were placed in roller tubes (2 per tube) – with the RNA-bound side facing inward – along with 20 mL of preheated ULTRAhyb. The tubes were then placed in a roller oven (VMR International, West Chester, PA) and the membranes were prehybridized at 42°C for 30 min at moderate rotation speed. During prehybridization, the radiolabeled probe solution was diluted 10-fold with 450 μL of 10mM EDTA and denatured in a heat block at 90°C for 10 min. The probe solution was then added to the hybridization buffer in the roller tubes and the probe hybridized with the RNA overnight (15-20 hr) at 42°C. Following hybridization, the hybridization buffer was discarded in an appropriate radioactive waste container. The membranes were washed twice with a low stringency solution (5 min/wash at room temperature) and twice with a high stringency solution (15 min/wash at 42°C). These washings were all discarded in the radioactive waste container. The membranes were then wrapped in plastic wrap, placed in cassettes with BIOMAX X-ray film (Kodak), and exposed for 1 hr to overnight (depending on the probe) at –80°C. To probe for additional RNAs, the membranes were then

stripped using two 15 min washes with hot 0.1X SSC, 0.1% lauryl sulfate (SDS; Fisher) solution (preheated to a boil).

Films were developed using a Kodak RD X-OMAT developer (Kodak) and scanned into 8-bit, grayscale TIFF images using a VXR-12 Film Digitizer (Vidar Systems Corp., Herndon, VA). Bands were analyzed by densitometry using the image processing program ImageJ v1.27 [National Institutes of Health, available at http://rsb.info.nih.gov/ij/]. The optical density of each mRNA band was corrected for background noise and normalized by the optical density of the corresponding 18S rRNA band.

4.2.7 *Histochemical staining*

After 31 days of culture wells were assayed for mineralization of the extracellular matrix using the von Kossa stain as previously described [Goldstein, 2001]. In order to test for osteocalcin secretion by the cell layers (not reported), osteogenic medium was replaced with serum-free osteogenic medium supplemented with 1 nM 1,25-dihydroxyvitamin D_3 (with or without 10 nM dexamethasone) two days prior to analysis. A 4% glutaraldehyde solution was prepared by diluting a 25% glutaraldehyde solution (Sigma) in sterile PBS. On day 31, wells were rinsed twice with 2 mL sterile PBS and the cell layers were fixed with 1 mL 4% glutaraldehyde solution to each well. After a 10-minute incubation, the glutaraldehyde was removed and the wells were rinsed twice with deionized water. A 0.5 mL volume of 5% $AgNO_3$ (Sigma) in water was added per well and the wells were exposed to incandescent light for approximately 30 minutes. The $AgNO_3$ solution was removed and the wells were washed twice with deionized water. To stain for cell membranes, one drop of safrinin-O (Fisher) was added to each well, the plates were agitated for a period of approximately 30 seconds, and the wells were then rinsed thrice with 100% ethanol to remove excess dye.

Culture images (12-bit, grayscale) were acquired at 4X using an Olympus model IX50 inverted microscope (Opelco, Sterling, VA) equipped with a Prior motorized stage accessory and a Hamamatsu cooled CCD camera (I-cube, Crofton, MD). Individual surface images were tiled to produce a single well image using ImagePro software (Media Cybernetics, Silver Spring, MD), as described in Appendix B-2. For each composite image, the histogram of the individual pixel intensities – from 0 (black) to 4095 (white) – had a bimodal distribution. (The first peak corresponded to dark areas of mineralized matrix, while the second corresponded to lighter

regions of cell layers that were stained with safrinin-O.) The histogram data for each well image was exported to Excel. The intensity corresponding to the minimum within the bimodal distribution was determined for each histogram. The average minimum intensity among all histograms was designated as the threshold to distinguish pixels associated with mineral deposits. Image Pro was used to identify objects (i.e., contiguous areas of mineralization) greater than 300 μm^2 within each well. Artifacts were deleted manually. The total area of mineral coverage within each well was determined by summing the areas of the individual objects.

4.2.8 *Statistics*

Unless noted elsewhere, measurements are presented as the mean ± standard error of the mean (S.E.M.) for 8 wells from two separate experiments (4 wells/experiment). A one-way analysis of variance in combination with Scheffe's multiple-comparison procedure was used to perform discrete pairwise comparisons of the means between treatment regimens (i.e., duration of dexamethasone supplementation). Statistical significance denotes a confidence of greater than 95% (α=0.05). Statistical analyses were performed using The SAS System for Windows release 8.02 (SAS Institute, Cary, NC), as described in Appendix A.

4.3 Results

4.3.1 Effect of dexamethasone treatment duration on cell number

Quantification of DNA indicated a decrease in cell number with increasing duration of dexamethasone treatment (Figure 4.1). At day 10, significantly fewer cells per well ($p<0.05$) were measured during continuous dexamethasone supplementation compared to discontinuation at day 3. After 14 days, a similar decrease in cell number ($p<0.05$) was associated with maintaining cells in dexamethasone-containing medium.

4.3.2 Effect of dexamethasone treatment duration on cell morphology

After initial seeding at 1×10^5 cells/well (~50% of confluence), the first passage cells attached to the surface and exhibited a fibroblast-like spindle shape. After 24 hours, the cells were supplemented with dexamethasone for varying durations. The cells reached confluency within a few days of secondary culture. As colonies merged into multicellular layers, there was a general transformation in cell morphology from spindle-like to polygonal in shape. However, a

difference in cell morphology among the different treatment regimens became more pronounced as the cultures matured (Figure 4.2). When dexamethasone was removed at earlier timepoints, cells were consistently smaller and more polygonal. This trend was more evident in regions of low cell density (i.e., where cell layers had begun to peel to form multilayered nodules). Adipocytes were observed in all cultures, as indicated by the presence of lipid vesicles in the cell colonies (Figure 4.2).

4.3.3 Effect of dexamethasone treatment duration on collagen synthesis

A slight increase in the rate of collagenous protein synthesis was measured with increasing duration of dexamethasone treatment at days 10 and 14 (Figure 4.3(a)). However, neither trend was statistically significant. Similar trends were also measured within the non-collagenase digestible fractions (data not shown), indicating a slight – though statistically insignificant – increase in total protein synthesis with continuous dexamethasone treatment. Consequently, the percent collagenous protein was not different among treatments (Figure 4.3(b)).

4.3.4 Effect of dexamethasone treatment duration on messenger RNA expression

Cells were subcultured in the presence of dexamethasone for 10, 17, or 24 days and assayed on day 24 for the expression of osteocalcin, bone sialoprotein, and lipoprotein lipase messenger RNAs and 18S ribosomal RNA. Markers for both the osteoblastic phenotype (osteocalcin, bone sialoprotein) and the adipocytic phenotype (lipoprotein lipase) were observed within these cultures (Figure 4.4). As expected, similar levels of 18S rRNA expression were measured among the samples. Although a slight increase in osteocalcin expression was observed among samples from a single study (lanes 2, 4, and 6), a replicate study revealed only trace levels of the mRNA. (Such batch-to-batch variability is not uncommon with cell derived from tissue explants.) However, Northern blot analysis indicated an increase in bone sialoprotein expression with continuous dexamethasone treatment. Conversely, a decrease in lipoprotein lipase expression was measured with increasing duration of treatment.

4.3.5 Effect of dexamethasone treatment duration on matrix mineralization

After 4 weeks in culture, histochemical staining revealed that cells formed multilayered colonies with varying degrees of mineral deposition (Figure 4.5(b)). The small gray spots indicate clustered cells – some of which had mineral deposits, while the larger black areas represent fully-mineralized nodules. The von Kossa stain demonstrated that wells with at least 17 days of dexamethasone supplementation yielded significantly higher areas of mineral deposition ($p<0.01$) compared to shorter treatment durations (Figure 4.5(a)). Maximum areas of mineralization were measured for 24 and 31 days of treatment ($p<0.01$), while negligible mineral deposition was observed when dexamethasone was removed at days 7 and 10.

4.4 Discussion

The purpose of this study was to examine the effects of dexamethasone treatment duration on the proliferation and differentiation of rat marrow stroma-derived osteoprogenitor cells. Removal of dexamethasone from the culture medium increased cell density, suggesting enhanced progenitor cell proliferation. A small but statistically insignificant increase in the rate of collagenous protein synthesis, a marker of extracellular matrix development, was measured with increasing duration of treatment. Phenotypic markers of osteoblastic and adipocytic differentiation were also examined to characterize culture development. While a change in osteocalcin mRNA expression could not be determined, an increase in the expression of bone sialoprotein was observed with increased duration of treatment. In contrast, lipoprotein lipase mRNA declined with increased treatment duration. Finally, the mineralization of extracellular matrix increased with prolonged steroid treatment.

In this study, osteoprogenitor cells were expanded in primary culture and subsequently introduced to dexamethasone in secondary culture. Removal of the glucocorticoid after initial supplementation was shown to increase cell density relative to continuous treatment. This effect suggests that dexamethasone inhibits proliferation, consistent with previous studies using human marrow stroma-derived osteoprogenitor cells that were also expanded in primary culture without the glucocorticoid [Cheng et al., 1994; Kim et al., 1999]. Furthermore, Kim et al. [1999] showed that the rate of proliferation – as measured by tritiated thymidine incorporation – was inhibited by dexamethasone in a dose-dependent manner. A variety of effects on proliferation have been reported when dexamethasone is immediately introduced to marrow explants. One study using

rat marrow stromal cells reported decreased proliferation in primary culture with the addition of steroid [Leboy et al., 1991]. Other studies have suggested either no change or an increase in proliferation [Atmani et al., 2002; Peter et al., 1998; Rickard et al., 1994]. Walsh et al. [2001] reported no detectable change in the proliferation of human marrow stromal cells at physiologically-equivalent dexamethasone levels, but an inhibition of proliferation at supraphysiological levels. The discrepancy in these results suggests that the effects of dexamethasone on osteoprogenitor cell proliferation may depend on their developmental stage, the donor-species, and the specific culture conditions.

Cell morphology was found to vary with the duration of dexamethasone treatment. With shorter periods of supplementation, the cells became increasing less spindle-like and more polygonal. This variation in cell morphology suggests that dexamethasone may alter either the synthesis of the surrounding extracellular matrix and/or the presentation of proteins that regulate cell adhesion. Cell-matrix adhesion is primarily regulated by integrins, heterodimeric transmembrane glycoproteins that consist of α and β subunits. These receptors transduce signals from the extracellular matrix to adherent cells through specific signaling pathways (e.g., mitogen-activated protein kinase pathways) [Clark and Brugge, 1995]. Dexamethasone has been reported to down-regulate expression of the α_2 and α_4 integrin subunits in primary human marrow stromal cell cultures [Walsh et al., 2001]. These subunits – when heterodimerized with the β_1 subunit – are known to bind with type I collagen ($\alpha_2\beta_1$), osteopontin ($\alpha_4\beta_1$), and fibronectin ($\alpha_4\beta_1$) [Grzesik and Robey, 1994]. Using both mature human osteoblasts and human marrow stromal cells, Cheng et al. [2000a] demonstrated that long-term exposure to dexamethasone inhibited the expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins, known receptors for osteopontin and vitronectin. Through interaction with integrin dimers, bone matrix proteins may regulate the sequential differentiation of osteoprogenitor cells *in vitro* [Adams and Watt, 1993; Mousi et al., 1997; Xiao et al., 1998].

Tritiated proline incorporation revealed an increase in the mean rate of collagenase-digestible protein synthesis with increasing duration of dexamethasone treatment, but this increase was not statistically significant. Further, the percent collagenous protein was not altered among the treatment regimens. These results differ from previous studies of both human and rat marrow stromal cells *in vitro*, in which dexamethasone was found to inhibit type I collagen expression and collagenous protein secretion [Beresford et al., 1992; Kim et al., 1999; Leboy et

al., 1991]. A decrease in collagen synthesis is consistent with observed decreases in proliferation, since collagen expression levels have been reported to peak during the proliferative stage of osteoblastic differentiation [reviewed by Lian and Stein, 1992]. A reciprocal relationship between the decline in proliferation and the subsequent onset of matrix maturation and mineral apposition has been characterized using fetal rat calvarial cells *in vitro* [Owen et al., 1990]. However, this study suggests that prolonged dexamethasone treatment enhances the secretion of markers for the mature osteoblastic phenotype, but not at the expense of collagen synthesis.

Expression of phenotypic markers for both osteoblastic (osteocalcin, bone sialoprotein) and adipocytic (lipoprotein lipase) development were observed after 24 days of subculture. An enhancement of osteoblastic differentiation with increasing duration of treatment was demonstrated by a marked increase in bone sialoprotein expression. The expression of osteocalcin may have also increased with prolonged steroid treatment, but more data is required for statistical inference. Such a result would be consistent with a recent study – using rat marrow stromal cells – which demonstrated maximal osteocalcin and bone sialoprotein expression with continuous dexamethasone supplementation [Atmani et al., 1992]. An increase in the expression of one or both markers has been reported with the general presence of dexamethasone in additional studies using rat marrow stromal cells [Aubin, 1999; Leboy et al., 1991; Kasugai et al., 1991; Rickard et al., 1994].

An increase in lipoprotein lipase expression (a marker of adipogenesis [Gimble, 1990]) was measured with decreasing duration of treatment. This trend may indicate that a portion of the osteoprogenitor cell population has differentiated along the adipocytic lineage following the discontinuation of dexamethasone treatment. Previous studies have proposed that osteoblasts and adipocytes differentiate from a common precursor in the bone marrow stroma [Bennett et al., 1991] and the degrees of adipogenesis and osteogenesis are reciprocal within rat marrow stromal cell cultures [Beresford et al., 1992]. Beresford et al. [1992] also demonstrated that the timing of dexamethasone treatment affected the relative levels of osteogenesis and adipogenesis. For this study, however, the culture images at day 14 do not demonstrate a clear difference in the number of adipocytes.

Analysis of the cell layers after four weeks demonstrated that maintained dexamethasone supplementation was required for maximal formation of mineral deposits. These results are

consistent with previous studies using rat marrow stromal cells and rat fetal calvaria cells, which have shown that continuous treatment with dexamethasone is required to maximize the formation of mineralized bone nodules [Aubin, 1999; Bellows et al., 1987; Beresford et al., 1992]. A study with human marrow stromal cells demonstrated that withdrawal of dexamethasone after 1 week of treatment significantly reduced the degree of mineralization [Cheng et al., 1994]. For our study, the mineralized area remained at relatively low levels for treatment durations of less than two weeks. After 17 days, there was a significant increase in coverage. This timepoint may correspond to the transition point between matrix maturation and mineralization, at which the cells have already secreted a collagen-based matrix, and the expression patterns change in favor of other matrix proteins such as osteocalcin and bone sialoprotein [Owen et al., 1990]. Dexamethasone may be required to stimulate the transition between these two stages.

The precise role of dexamethasone in osteoblastic differentiation has been masked by study-to-study differences in the response of osteoprogenitor cells to this glucocoritocoid. These variations may be attributed to differences in the culture conditions, including the species/age/sex of the cell donor, the donor's physiological status, the anatomical source of harvested progenitor cells (e.g., marrow stromal cells, calvarial cells), the maturation of the cells studied (i.e., the duration of primary and secondary cultures), the cell seeding density, the combination of osteogenic supplements in the culture medium, and the timing of glucocorticoid supplementation (primary culture vs. secondary culture). These conditions define the subpopulation composition of the culture. Aubin [1999] proposed that heterotypic cell-cell interactions may influence osteogenesis in marrow stromal cells. Furthermore, dexamethasone may act through nonosteogenic cell types – including hematopoietic cells – to affect osteoprogenitor differentiation [Aubin, 1999; Herbertson and Aubin, 1995]. In this study, cells were not exposed to dexamethasone until secondary culture. The lack of heterotypic cell interactions in secondary culture may alter the measured responses to glucocorticoid treatment when compared to cultures in which glucocorticoid was supplemented immediately to harvested cells. Furthermore, the adherent cell population isolated in primary culture may not retain their osteogenic capacity during clonal expansion in the absence of dexamethasone. A previous study reported that the effects of glucocorticoids on fetal rat calvarial cell morphology and gene expression were dependent on the developmental stage of cells when steroid was first introduced.

The results indicate that dexamethasone inhibits the proliferation of osteoprogenitor cells in secondary culture. However, continuous supplementation with the glucocorticoid is required for maximal mineralization. Together these results suggest that dexamethasone is required to direct the cells from a proliferative but undifferentiated state toward terminal maturation. Although previous studies have shown that dexamethasone down-regulates type I collagen [Beresford et al., 1992; Kim et al., 1999; Leboy et al., 1991], the primary structural protein in bone matrix, in this study we find that its overall benefits for the development of mineralized bone appear to outweigh the reported negative effects on matrix synthesis. In the future, marrow stroma-derived progenitor cells may be coupled with biodegradable scaffolds for applied bone augmentation and defect repair. In order to optimize the therapeutic capacity of such grafts, the progenitor cells may need to be directed along the osteoblastic lineage to a specific stage prior to implantation [Bruder et al., 1998]. The duration of glucocorticoid supplementation *in vitro* may be a key factor in controlling the extent of differentiation.

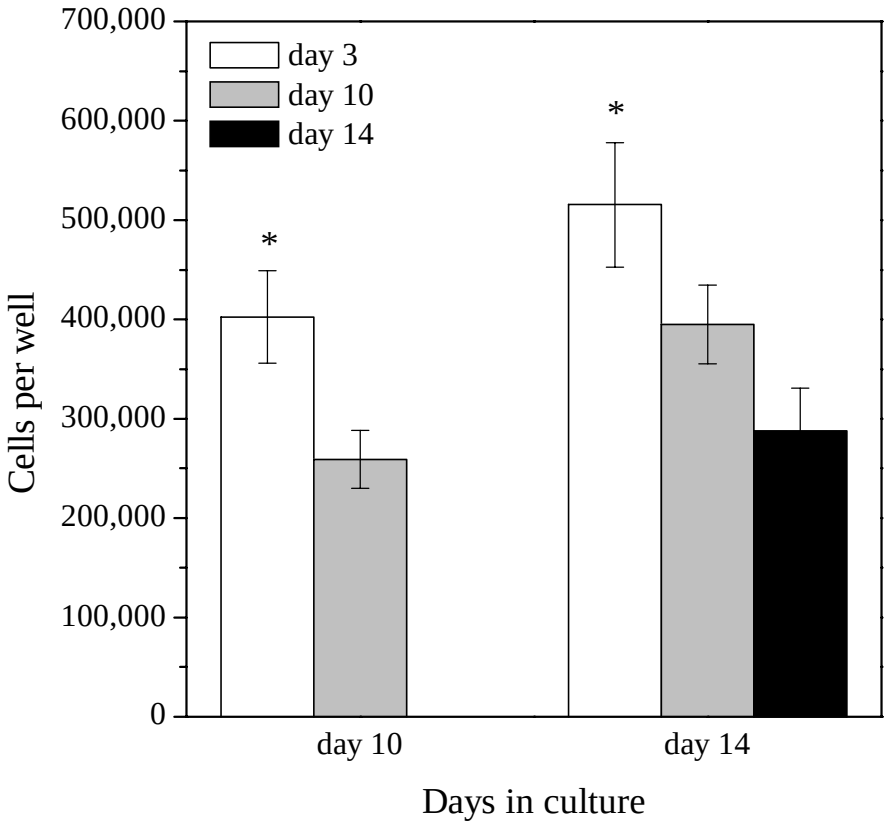

Figure 4.1: Cell density determined by fluorometric quantification of DNA. The number of cells per well are plotted versus subculture duration. Culture medium was supplemented with dexamethasone for 3 (white), 10 (gray), or 14 (black) days. Each bar represents the mean ± S.E.M. for 8 wells (over two separate experiments). A single asterisk (*) denotes statistically significant difference from continuous treatment ($p<0.05$).

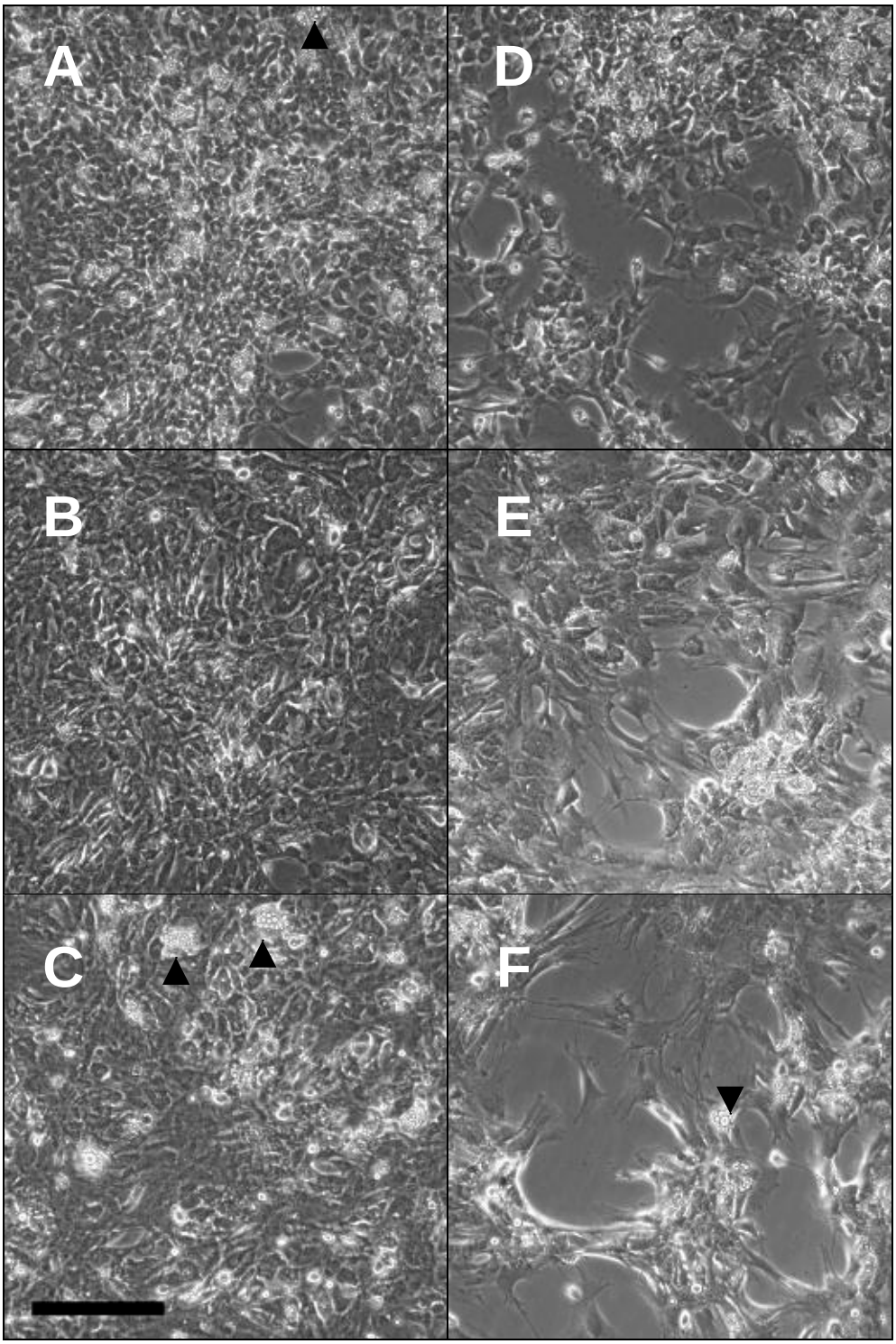

Figure 4.2: Cell morphology after 14 days in culture. Subcultured rat marrow stromal cells were supplemented with dexamethasone for either 3 (A,D), 10 (B,E), or 14 (C,F) days. Phase contrast images were taken for areas with relatively high (A-C) and low (D-F) degrees of confluence. Arrowheads indicate lipid vesicles (i.e., adipocytes). Scalebar = 200 μm.

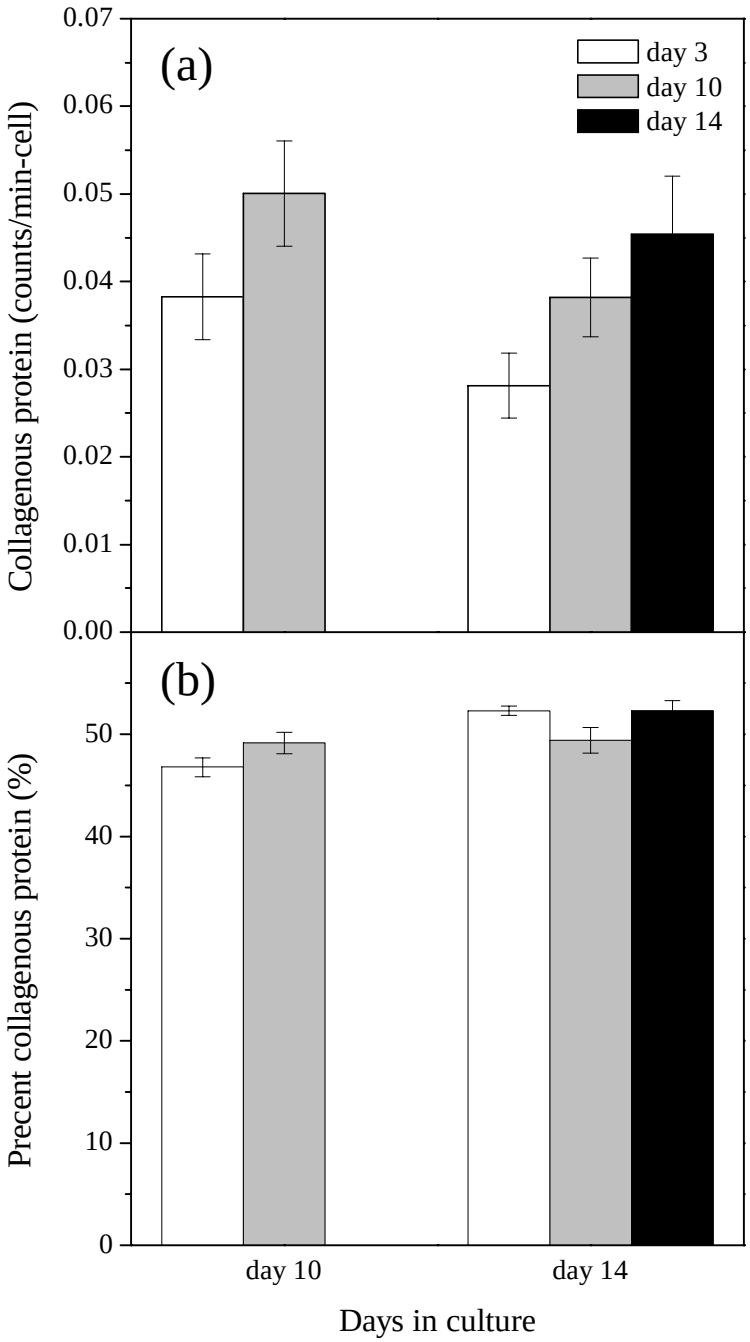

Figure 4.3: Collagen synthesis determined by ^{3}H-proline incorporation. (a) Counts per minute from collagenase-digestible protein fractions normalized by mean cell number (determined in Figure 4.1) and (b) the collagenase-digestible protein as a percentage of total protein synthesized are plotted versus subculture duration. Culture medium was supplemented with dexamethasone for 3 (white), 10 (gray), or 14 (black) days. Each bar represents the mean ± S.E.M. for 8 well samples (over two separate experiments).

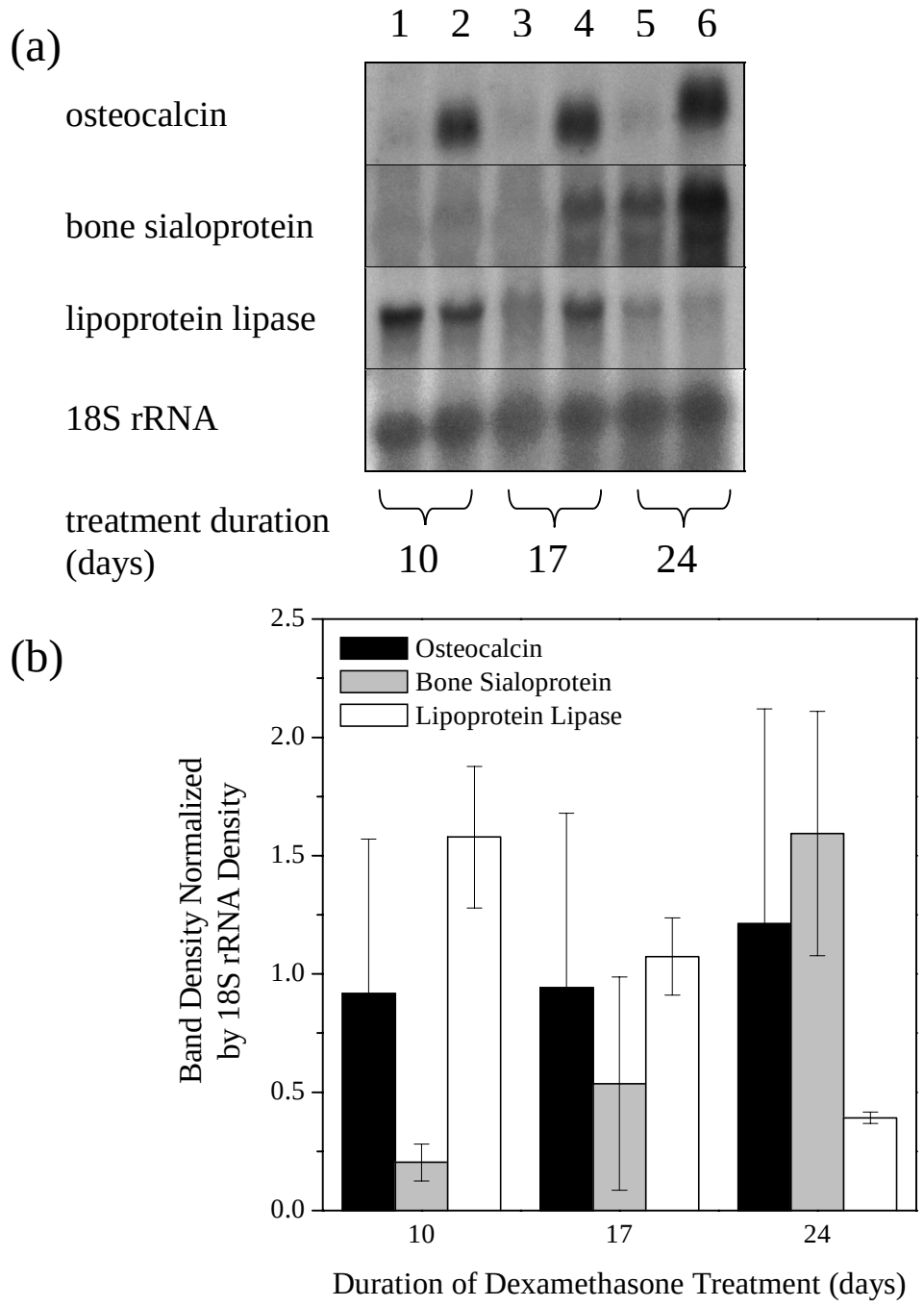

Figure 4.4: Protein expression as determined by Northern blot analysis. (a) Bands for osteocalcin mRNA, bone sialoprotein mRNA, lipoprotein lipase mRNA, and 18S rRNA are presented for cells cultured for 24 days in medium supplemented with dexamethasone for either 10, 17, or 24 days. Each band represents the RNA from 4 well samples (from the same tissue-culture plate). Lanes 1, 3, and 5 are from a single 12-well plate, while lanes 2, 4, and 6 are from a replicate plate. (b) Band densities (relative to 18S rRNA band densities) for osteocalcin (black), bone sialoprotein (gray), and lipoprotein lipase (white) mRNAs are plotted versus dexamethasone treatment duration. Each bar represents the mean of 2 replications, and each error bar denotes the range between replicates.

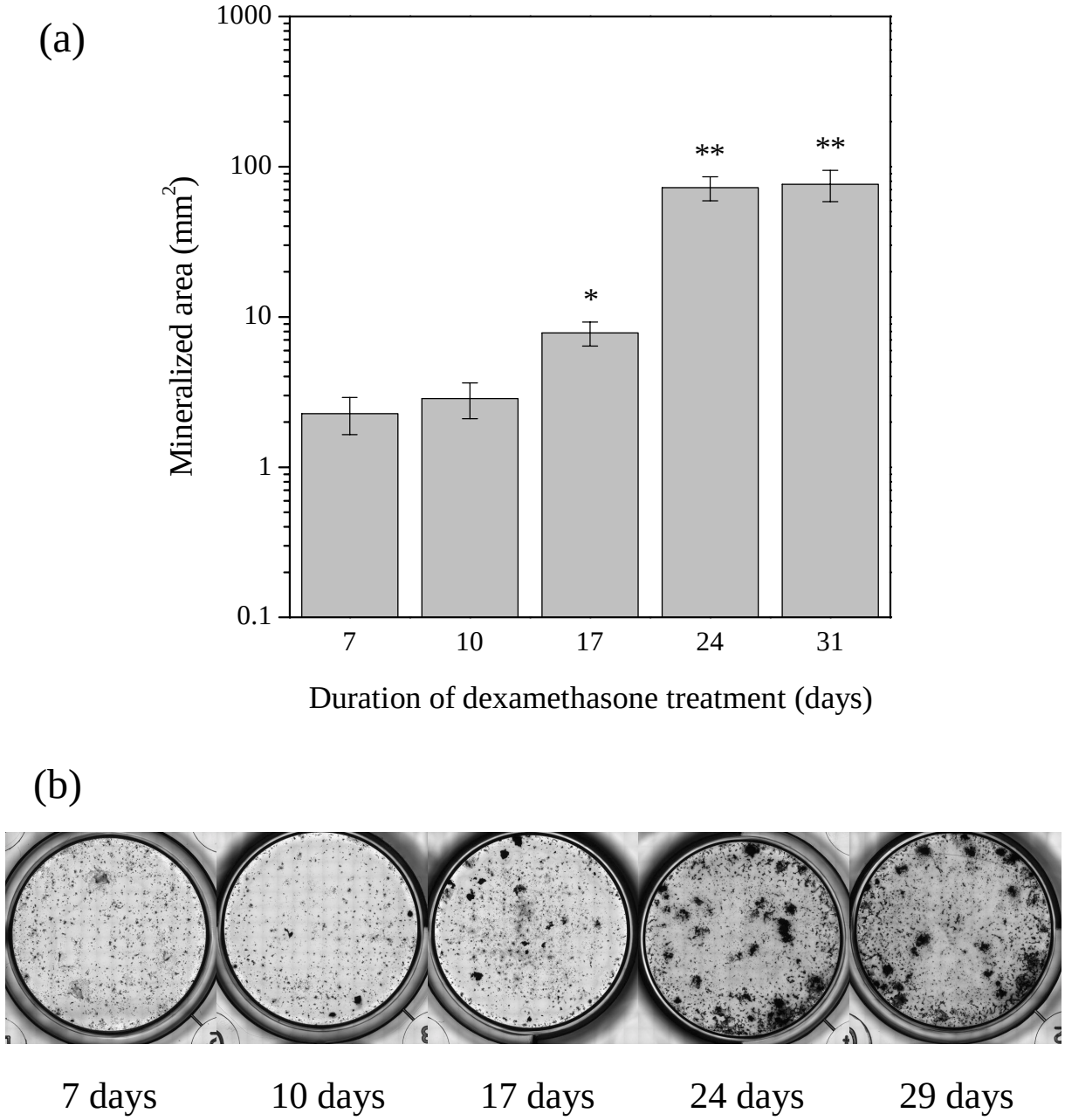

Figure 4.5: Areas of mineralization as determined by von Kossa silver stain. (a) Area of mineral deposit per well is plotted versus the dexamethasone treatment duration. Each bar represents the mean and standard error of 8 wells (over two separate experiments). A single asterisk (*) denotes statistically significant difference from day 3 removal ($p<0.001$). A double asterisk (**) denotes statistically significant difference from day 17 removal ($p<0.001$). (b) Representative wells for each treatment are presented. Areas of mineral deposit appear black from the von Kossa stain. Small cell clusters appear gray.

Chapter 5:
Conclusions

5.1 Summary

The central purpose of this thesis project was to quantitatively analyze glucocorticoid action on the differentiation of bone marrow stromal progenitor cells both *in vivo* and *in vitro*. The studies presented in Chapters 2 and 3 were undertaken in order to relate changes in the osteoprogenitor cell population to the pathogenesis of glucocorticoid-induced bone disease, while the purpose of Chapter 4 was to evaluate dexamethasone as a supplement for osteoinduction *in vitro*. Specifically, the three primary research aims were (1) to develop and characterize a rat model for glucocorticoid-induced osteopenia and marrow adipogenesis, (2) to identify any changes in the capacity of bone marrow explants – isolated from glucocorticoid-treated rats and cultured *in vitro* – to express markers of the osteoblastic phenotype, and (3) to examine the effects of intermittent dexamethasone treatment on the differentiation of osteoprogenitor cells isolated from healthy rat marrow (i.e., marrow not treated with glucocorticoids *in vivo*).

Methylprednisolone treatment (18 mg/kg/day for up to 42 days) produced a rat model with increased volume of fatty (yellow) marrow and reduced radial bone growth. However, no detectable differences in femoral wall thickness, mineral density, or ultimate shear stress were measured. In growing rats, cortical bone is formed in concentric lamellae on the periosteal (outer) surface by osteoblasts and simultaneously resorbed along the endosteal (inner) surface by osteoclasts [Currey, 1984]. The experimental results suggest that high glucocorticoid levels inhibit both bone formation and resorption without significantly altering the balance between these two processes. Previous rat studies demonstrated significantly decreased bone growth, strength, and mineral density following subcutaneous injection with 1 mg/kg/day methylprednisolone or 5 mg/kg/day prednisolone for 90 or 80 days, respectively [Ørtoft and Oxlund, 1988; Ørtoft and Oxlund, 1996]. Therefore, either lower doses or higher durations of glucocorticoid treatment may induce a marked osteopenic condition within rats.

Primary culture of bone marrow explants (minus buoyant yellow marrow) from treated rats did not demonstrate any marked differences in their potential to form multilayered cell colonies (nodules) that mineralize and express phenotypic markers of osteoblast maturation (i.e.,

osteogenic potential). However, a decrease in the absolute number of nucleated marrow cells (i.e., in the volume of red marrow) suggests a decrease in the total osteogenic potential of the bone marrow. This reduction in the red marrow volume might be attributed to both decreased bone growth and increased yellow marrow volume (both described in chapter 2). We proposed that, at supra-physiological levels, glucocorticoids might irreversibly recruit a fraction of the osteoprogenitor cells to differentiate into the adipogenic lineage. The observed increase in yellow marrow volume and concurrent decrease in red marrow volume is consistent with this hypothesis. However, osteoprogenitor cells from the red marrow of treated rats were as capable of producing bone-like tissue as control cells when introduced to osteogenic supplements *in vitro*. Furthermore, a recovery in the marrow properties was observed following cessation of treatment. Glucocorticoid-induced bone loss has been partially attributed to the direct effects of these steroids on cells of the osteoblastic lineage, inhibiting their number and metabolic activity [reviewed by Canalis, 1996; Weinstein and Manolagas, 2000]. The results from this study suggest that supra-physiological glucocorticoid levels do not affect either the relative number of marrow progenitor cells or their responsiveness to osteogenic stimuli. Instead, glucocorticoid administration may suppress the osteoblastic differentiation of their clonogenic progeny in favor of adipogenesis.

After isolation and expansion of adherent marrow stromal cells (from untreated rats) in primary culture, intermittent dexamethasone treatment (10^{-8} M) in secondary culture demonstrated that continuous supplementation maximized the deposition of bone-like mineral. When dexamethasone treatment was discontinued during the proliferative stage, cell density increased, suggesting that dexamethasone directs the osteoprogenitor cells from proliferation to differentiation. Discontinuation of dexamethasone treatment during the matrix development stage reduced the expression of bone sialoprotein, an osteogenic marker, while concurrently increasing the expression of lipoprotein lipase, an adipogenic marker. These effects on protein expression indicate an alteration in progenitor cell differentiation that favors adipogenesis. Thus, dexamethasone may be required not only for progenitor cell recruitment along the osteoblastic lineage, but also to further direct these cells into mature osteoblasts. Dexamethasone has been shown to be an effective but nonspecific inductor of osteoblastic differentiation [Beresford et al., 1992; Maniatopoulos et al., 1988]. Continuous treatment with physiologically equivalent levels

of dexamethasone appears to enhance osteoblastic differentiation of marrow stromal cells *in vitro*.

These three studies collectively demonstrate that glucocorticoids have concentration- and time-dependent effects on the differentiation of bone marrow stromal progenitor cells both *in vivo* and *in vitro*. While prolonged treatment with supra-physiological levels of glucocorticoids inhibits osteoblastic differentiation and concurrently enhances adipocytic differentiation *in vivo*, continuous supplementation with physiologically-equivalent concentrations *in vitro* was shown to direct differentiation in favor of the osteogenic pathway (Figure 5.1). The results indicate the importance of steroid action on marrow stromal progenitor cells in the pathogenesis of glucocorticoid-induced bone disease. Future research into the constitutive mechanisms of these disorders – and their potential therapies – should focus on these cells and their differentiation pathways. In addition to bone disease, the implications of our work may also extend to bone tissue engineering. For example, one current goal in orthopaedic research is to develop synthetic bone grafts by utilizing small marrow explants containing autogenous osteoprogenitor cells. These cells can be expanded *in vitro*, seeded into biodegradable scaffolds, cultured under osteoinductive conditions, and finally implanted at the site of a significantly large defect. Glucocorticoids (e.g., dexamethasone) have become a standard supplement for the differentiation of osteoprogenitor cells *in vitro*. In fact, previous studies have suggested that these steroid hormones are necessary for such differentiation. For our study, cessation of dexamethasone treatment *in vitro* not only inhibited the progression of osteogenesis, but also increased adipogenesis.

5.2 Future Work

5.2.1 *Improvements to the rat model for glucocorticoid-induced osteopenia*

The glucocorticoid treatment regimen used for this study (18 mg/kg/day of methylprednisolone) did not produce a rat model with diminished bone mineral density and ultimate shear stress. A decrease in radial bone growth was observed, but without a concurrent decrease in femoral wall thickness. The results suggest that glucocorticoid treatment inhibited both osteoblastic bone formation and osteoclastic resorption. To confirm these results, histological staining for viable osteoblasts and osteoclasts along the trabecular and cortical

surfaces should be performed. Furthermore, future studies should explore the possibility of localized trabecular bone loss (e.g., in the femoral metaphysis).

Ørtoft and Oxlund observed significantly decreased bone growth, strength, and mineral density in rats following subcutaneous injection with either 1 mg/kg/day methylprednisolone [1988] or 5 mg/kg/day prednisolone [1996] for up to 90 days (methylprednisolone) or 80 days (prednisolone). However, in an additional study, no significant effects on cortical bone were observed following subcutaneous injection with 5 mg/kg/day methylprednisolone for 30 days [Ørtoft et al., 1992]. Therefore, to create an osteopenic rat model, future studies may require longer durations of glucocorticoid treatment. Furthermore, blood serum levels of glucocorticoid should be measured to assess the extent of intestinal absorption following administration by oral gavage. If blood serum levels are inadequate, an alternative administration technique may be required (e.g., subcutaneous injection).

In this study, methylprednisolone treatment inhibited weight gain and likely made the rats lethargic. A state of lethargy may limit the rats' movement and consequently reduce mechanical stimulation (i.e., cyclic bending strains) of the hindlimbs. Since mechanical stimulation of bone by climbing [Notomi et al., 2001], jumping [Notomi et al., 2000; Umemura et al., 1997], and treadmill running [Iwamoto et al., 1999; Mosekilde et al., 1994] has been shown to increase bone mass in growing rats, and skeletal unloading through tail suspension [Bikle et al., 1987; Globus et al., 1986] and sciatic neurectomy [Weinreb et al., 1989; Zeng et al., 1996] has been shown to decrease bone mass in rats, differences in bone and bone marrow properties may be partially attributed to a lethargic state. To minimize the effects of lethargy, rats in future studies should be subjected to a regulated exercise regimen. For example, rats could be required to climb ladders or blocks in order to reach their food and water supplies. Alternatively, both treated and untreated rats could be placed on a treadmill apparatus.

5.2.2 *Identification of changes in the osteogenic capacity of bone marrow stromal cells*

Moderate differences in the osteogenic capacity of marrow stromal cells may have been masked by large culture-to-culture variances in the measured responses (see section 3.3). These variances may be attributed to the heterogeneous nature of primary bone marrow stromal cell cultures, which initially contain both hematopoietic and mesenchymal cell lines. The density of osteoprogenitor cells should be increased in future studies in order to minimize the heterogeneity

of the experimental model. Previous studies have reported that osteoprogenitor cells only make up about $1/10^5$ of the total nucleated marrow cells seeded onto tissue culture polystyrene [Aubin, 1999; Rickard et al., 1996], which corresponds to a seeding density of about 80 osteoprogenitor cells per well for our study. Enhanced mineralization has been associated with increasing osteoprogenitor seeding density [Aubin, 1999, Herbertson and Aubin, 1995; Jaiswal et al., 1997]. In one recent study, rat osteoprogenitor cells were seeded as dense clusters in secondary culture, resulting in large nodules that were heavily mineralized [Goldstein, 2001]. These results support the hypothesis that cell-cell interactions are important in osteoblastic differentiation [Lecanda et al., 1998; Cheng et al., 1998]. Increasing the initial density of osteoprogenitor cells in culture should decrease the variation in their differentiation.

If significant differences in the osteogenic potential of marrow stromal cells had been observed between treated and untreated rats, the current set of analyses would not distinguish between glucocorticoids effects on absolute osteoprogenitor cell number and on osteoprogenitor cell responsiveness (i.e., to osteogenic medium *in vitro*). Future studies should implement assays to measure initial osteoprogenitor cell number in order to make such a distinction. Recent studies have identified an antigen, STRO-1, that may be specific to multipotential precursor cells in the human marrow stroma [Dennis et al., 2002; Gronthos et al., 1994; Simmons and Torok-Storb, 1991]. If a similar rat antigen could be identified, a monoclonal antibody for this antigen could be used to measure the initial number of osteoprogenitor cells in our marrow stromal cell cultures. This number could then be used to normalize the responses from the corresponding mature cultures.

5.2.3 *Evaluation of dexamethasone as a supplement for osteoblastic differentiation in vitro*

In this study, continuous dexamethasone treatment of rat bone marrow stromal cells in secondary culture was shown to enhance expression of the osteoblastic phenotype. However, previous studies have demonstrated that dexamethasone treatment in both primary and secondary culture further enhances osteoblastic differentiation when compared with treatment in either passage alone [Aubin, 1999; Beresford et al., 1992; Ter Brugge and Jansen, 2002]. This observation suggests that there is a loss of osteogenic potential during primary culture without dexamethasone. We offer two possible explanations for this phenomenon. First, the absence of osteogenic supplements in primary culture may cause a fraction of the osteoprogenitor cells to

differentiate along a parallel mesenchymal lineage, such as the adipogenic lineage. Second, Aubin [1999] proposed that dexamethasone may act through nonosteogenic cells (e.g., those of the hematopoietic lineage) to affect osteoprogenitor differentiation. Since most of these non-adherent cells are removed during primary culture, the immediate presence of dexamethasone may further enhance osteoprogenitor cell differentiation though heterotypic cell interactions that are not available in secondary culture. Regardless of the cause, future studies using dexamethasone in primary culture may be required to confirm our conclusion that removal of dexamethasone in secondary culture inhibits expression of the osteoblastic phenotype.

A qualitative change in cell morphology was observed with varying dexamethasone treatment duration (as described in section 4.3.2). Cells treated continuously with the glucocorticoid were more cuboidal and formed tight clusters (the focal points of mineral deposition). These morphological changes may demonstrate an alteration in the synthesis of membrane proteins that regulate cell-cell (e.g., cadherins) and cell-matrix (e.g., integrins) adhesion. Furthermore, the presence of localized mineralization may indicate that dexamethasone affects communication between juxtapose cells by regulating the presentation of connexins (the constitutive subunits of gap junctions) on the cell surface. Gap junction-mediated communication has been shown to play an important role in osteoblastic differentiation [Lecanda et al., 1998; Schiller et al., 2001]. However, to our knowledge, no studies have looked specifically at the effects of dexamethasone on the expression of connexins 43 and 45, the primary gap junction subunits found in osteoblasts. Thus a set of studies that examines such effects – as well as any subsequent changes in intercellular signaling – may reveal an important mechanism by which dexamethasone regulates osteoprogenitor cell differentiation.

5.3 Concluding Remarks

Glucocorticoids have potent time- and concentration-dependent effects on the cells responsible for remodeling the musculoskeletal system. Efforts to identify the mechanisms by which they regulate tissue development are ongoing. These efforts may contribute to the development of therapies for steroid-related disorders *in vivo* and treatment strategies for bone tissue engineering *in vitro*.

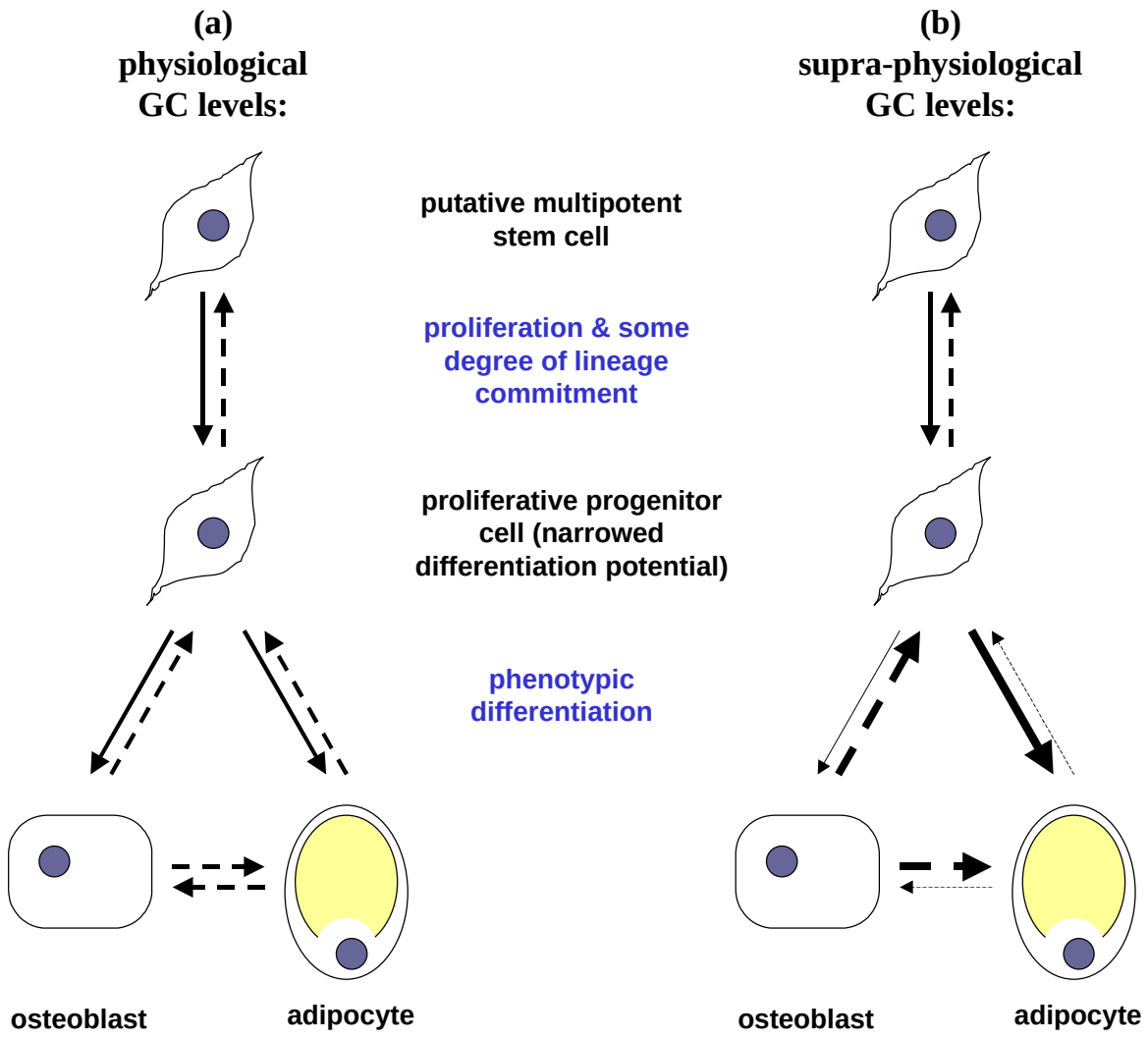

Figure 5.1: Glucocorticoid action on osteoprogenitor cell differentiation. The precise pathways involved in osteogenesis and adipogenesis are complex and not well understood. Dashed arrows represent pathways that have not yet been proven. The direct precursors for osteoblasts and adipocytes may be derived from putative multipotent stem cells found in the bone marrow stroma. (a) In the presence of physiologically-equivalent glucocorticoid concentrations, a balance between osteogenesis and adipogenesis is maintained. (b) However, supra-physiological levels enhance adipogenesis while diminishing osteogenesis (represented by differences in arrow thickness). The results of our studies suggest that these effects occur downstream of the undifferentiated progenitor cells.

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Appendix A:

Statistical analysis using SAS

Two different statistical analyses were performed using The SAS System for Windows release 8.02 (SAS Institute, Cary, NC). First, to compare means among treatments (e.g., glucocorticoid treatment duration *in vivo*, dexamethasone treatment duration *in vitro*), a one-way analysis of variance (ANOVA) was used in combination with Scheffe's multiple comparisons procedure. An example portion of SAS code and the corresponding output are provided below, with the pertinent output p-values and mean groupings (for Scheffe's procedure) highlighted in bold blue:

CODE:

```
title '*** Statistical Analysis ***';
title2 'Nucleated marrow cells for in vitro study';

options ls=77 ps=56 nodate nonumber;

title3 'ANOVA test for recovery from dex, dosed rats';

data recovery_dosed;
input trt $ cells;
lines;
day42  22264000
day42  45080000
day42  39936000
day42  50304000
day70  72960000
day70  117120000
day70  61440000
day70  63360000
day70  98880000
day70  64320000
;
run;

proc glm data=recovery_dosed;
class trt;
model cells = trt;
means trt / scheffe;
run;
```

OUTPUT:

```
                    *** Statistical Analysis ***
                Nucleated marrow cells for in vitro study
              ANOVA test for recovery from dex, dosed rats
```

The GLM Procedure

Class Level Information

Class	Levels	Values
trt	2	day42 day70

Number of observations 10

*** Statistical Analysis ***
Nucleated marrow cells for in vitro study
ANOVA test for recovery from dex, dosed rats

The GLM Procedure

Dependent Variable: cells

Source	DF	Sum of Squares	Mean Square	F Value	**Pr > F**
Model	1	3.8947216E15	3.8947216E15	10.07	**0.0131**
Error	8	3.0956109E15	3.8695137E14		
Corrected Total	9	6.9903325E15			

R-Square	Coeff Var	Root MSE	cells Mean
0.557158	30.94572	19671079	63566400

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	1	3.8947216E15	3.8947216E15	10.07	0.0131

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	1	3.8947216E15	3.8947216E15	10.07	0.0131

*** Statistical Analysis ***
Nucleated marrow cells for in vitro study
ANOVA test for recovery from dex, dosed rats

The GLM Procedure

Scheffe's Test for cells

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	3.87E14
Critical Value of F	5.31766
Minimum Significant Difference	2.93E7
Harmonic Mean of Cell Sizes	4.8

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

Scheffe Grouping	**Mean**	**N**	**trt**
A	**79680000**	**6**	**day70**
B	**39396000**	**4**	**day42**

Secondly, to test for time-dependent trends over days 0-42 or days 0-72, a simple linear regression model was used (null hypothesis: slope = 0 for a linear model). An example portion of SAS code and the output are provided below, with the estimated slope and corresponding p-value highlighted in bold blue:

CODE:

```
title '*** Statistical Analysis ***';
title2 'Nucleated marrow cells for in vitro study';

options ls=77 ps=56 nodate nonumber;
goptions aspect = 5;

title3 'ANOVA test for trends, days 0-42, dosed rats';

data dosed42;
label cells = 'available cells' duration ='duration (days)';
input trt $ duration cells;
lines;
D  0  35328000
D  0  38016000
D  0  82532000
D  0  68996000
D  14  63210000
D  14  60270000
D  14  50304000
D  14  24000000
D  28  37632000
D  28  37436000
D  28  39360000
D  28  58560000
D  42  22264000
D  42  45080000
D  42  39936000
D  42  50304000
;
run;

proc reg data=dosed42;
model cells = duration / clb;
output out=outreg pred=pred
       lclm=lclm uclm=uclm
       lcl=lcl ucl=ucl;
run;
```

OUTPUT:

```
                          *** Statistical Analysis ***
```

Nucleated marrow cells for in vitro study
ANOVA test for trends, days 0-42, dosed rats

The REG Procedure
Model: MODEL1
Dependent Variable: cells available cells

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	6.421844E14	6.421844E14	2.69	0.1235
Error	14	3.347395E15	2.390996E14		
Corrected Total	15	3.989579E15			

Root MSE	15462847	R-Square	0.1610
Dependent Mean	47076750	Adj R-Sq	0.1010
Coeff Var	32.84604		

Parameter Estimates

Variable	Label	DF	**Parameter Estimate**	Standard Error	t Value	**Pr > |t|**
Intercept	Intercept	1	55576500	6468573	8.59	<.0001
duration	duration (days)	1	**-404750**	246971	-1.64	**0.1235**

Parameter Estimates

Variable	Label	DF	95% Confidence Limits	
Intercept	Intercept	1	41702791	69450209
duration	duration (days)	1	-934451	124951

Appendix B:

ImagePro subroutines

B-1: Densitometry for RT-PCR analysis

The following pair of subroutines was written – using the macro editor in ImagePro – for the purpose of measuring the optical density of bands from images of agarose gels:

CODE: (comments are marked in violet)

```
Global bandx0 As Integer, bandy0 As Integer, bandw As Integer, bandh As
Integer
'The above line defines four AOI variables as global variables for use in
SelBand and Dens subroutines
Sub SelBand() 'Select band for densitometry
      Dim ImID1 As Integer, ImID2 As Integer
      ret = IpDocGet(GETACTDOC, 0, ImID1)
      'Variables bandw and bandh represent the width and height of the AOI
      which will be used to measure optical density
      bandw = 68
      bandh = 24
      'Variables bandx0 and bandy0 represent the x and y coordinates of the
      upper-left corner of the AOI
      bandx0 = 813
      bandy0 = 402
      'The next line creates an new image for the AOI to be pasted into
      (allows for quick check of AOI at 100% zoom)
      ImID2 = IpWsCreate(bandw, bandh, 100, IMC_GRAY)
      ret = IpAppSelectDoc(ImID1)
      'Actual definition steps for the AOI
      ipRect.Left = bandx0
      ipRect.top = bandy0
      ipRect.Right = ipRect.Left + bandw
      ipRect.bottom = ipRect.top + bandh
      ret = IpAoiCreateBox(ipRect)
      ret = IpWsCopy()
      ret = IpAppSelectDoc(ImID2)
      ret = IpWsPaste(0, 0)
      ret = IpAppSelectDoc(ImID1)
End Sub

Sub Dens() 'Densitometry analysis of gel bands
      Dim i As Integer, j As Integer, m As Integer, n As Integer
      Dim ImID1 As Integer, ImID2 As Integer, ImID3 As Integer
      ret = IpDocGet(GETACTDOC, 0, ImID1)
      ret = IpAppSelectDoc(ImID1)
      ipRect.Left = bandx0
      ipRect.top = bandy0
      ipRect.Right = ipRect.Left + bandw
      ipRect.bottom = ipRect.top + bandh
      ret = IpAoiCreateBox(ipRect)
```

```
      'The next line creates a histogram of the pixel intensities
      ret = IpHstCreate()
      'The next line exports the histogram data to Excel
      ret = IpHstSave("", S_DDE)
      ret = IpHstSelect(0)
      ret = IpHstDestroy()
      ret = IpAppSelectDoc(ImID1)
End Sub
```

B-2: Collecting tiled images for mineralization analysis

The following subroutine was written – using the macro editor in ImagePro – for the purpose of collecting individual culture images (at 4X magnification), tiling these into a single image for the entire well, and exporting a histogram of the individual pixel intensities to Excel:

CODE: (comments are marked in violet)

```
Sub WellImage() 'Collects of a tiled well image
      Dim a As Integer, b As Integer, c As Integer, d As Integer
      Dim e As Integer, f As Integer, g As Integer, h As Integer
      Dim i As Integer, j As Integer, k As Integer, m As Integer
      Dim n As Integer, p As Integer, q As Integer, r As Integer
      Dim s As Integer, t As Integer, deltax As Integer, deltay As Integer
      Dim Combined As Integer
      t=13
      ReDim ImID(1 To t) As Integer
      Dim ImInfo As IPDOCINFO
      deltax=11.9943
      deltay=11.9967
      r=1
      ret = IpStageXY(0, -deltay)
      ipStgVal = STG_CURRENT
      ret = IpStageControl(SETORIGIN, ipStgVal)
      'This next command collects 4X images by moving the stage in increments
      along an array that covers the entire well surface
      ret = IpStageAcq("\\", "\\", 0)
      ret = IpStageXY(0, deltay)
      ipStgVal = STG_CURRENT
      ret = IpStageControl(SETORIGIN, ipStgVal)
      'Retreiving document information and assigning the document to ImID1
      ret = IpDocGet(GETACTDOC, 0, ImID(r))
      ret = IpDocGet(GETDOCINFO, ImID(r), imInfo)
      a=14 'number of tile columns
      b=14 'number of tile rows
      'This next line creates a new image and defines it as ImID2
      ImID(r+1) = IpWsCreate(imInfo.width, ImInfo.height, 100, IMC_GRAY12)
      ret = IpAppSelectDoc(ImID(r))
      c = ImInfo.width/a
      d = ImInfo.height/b
      'This For loop will copy tiles, rotate them, and paste them into the
      same x, y position of the new document
```

```
      f = 0
      For g=1 To a
            e = 0
            For h=1 To b
                  ipRect.Left = e
                  ipRect.top = f
                  ipRect.Right = e+c-1
                  ipRect.bottom = f+d-1
                  ret = IpAoiCreateBox(ipRect)
                  'Rotates tile by 180 degrees before copying and assigns new
                  image to ImID3
                  ImID(r+2) = IpWsOrient(OR_ROTATE180)
                  ret = IpWsCopy()
                  ret = IpAppSelectDoc(ImID(r+1))
                  ret = IpWsPaste(e, f)
                  'Closes image created by IpWsOrient command
                  ret = IpDocCloseEx(ImID(r+2))
                  ret = IpAppSelectDoc(ImID(r))
                  e = e+c
            Next h
      f = f+d
      Next g
      ret = IpAppSelectDoc(ImID(r+1))
      'This next lines calls for a histogram of the combined image
      ret = IpHstCreate()
      'This next line exports the histogram data into Excel
      ret = IpHstSave("", S_DDE)
End Sub
```

B-3: Analysis of fatty marrow fractions

The following subroutine was written – using the macro editor in ImagePro – for the purpose of measuring areas of healthy and fatty marrow within images of mid-diaphysiseal cross sections (stained with hematoxylin and eosin) and exporting the data to Excel:

CODE: (comments are marked in violet)

```
Sub marrow_areas() 'Measures areas of healthy and fatty marrow
      ret = IpBlbShow(1)
      ret = IpSegPreview(0)
      ret = IpSegShow(1)
      ret = IpSegSetAttr(SETCURSEL, 0)
      ret = IpSegSetAttr(CHANNEL, 0)
      'These next lines defines the range of intensities for Red, Green, and
      Blue that be used to select for regions of healthy marrow (0 = Red, 1 =
      Green, 2 = Blue)
      ret = IpSegSetRange(0, 0, 255)
      ret = IpSegSetAttr(CHANNEL, 1)
      ret = IpSegSetRange(1, 0, 120)
      ret = IpSegSetAttr(CHANNEL, 2)
      ret = IpSegSetRange(2, 0, 255)
```

```
	ret = IpSegShow(0)
	ret = IpBlbUpdate(1)
	'This next line defines the range of areas for object of interest
	(values are in square pixels)
	ret = IpBlbSetFilterRange(BLBM_AREA, 267.8060303, 8926868.0)
	'This next line defines the setting for the count/measure tool
	ret = IpBlbSetAttr(BLOB_MEASUREOBJECTS, 1)
	ret = IpBlbSetAttr(BLOB_FILTEROBJECTS, 1)
	ret = IpBlbSetAttr(BLOB_CLEANBORDER, 0)
	ret = IpBlbSetAttr(BLOB_FILLHOLES, 0)
	ret = IpBlbSetAttr(BLOB_LABELMODE, 0)
	ret = IpBlbSetAttr(BLOB_OUTLINEMODE, 1)
	'This next line initiates the count/measure tool
	ret = IpBlbCount()
	ret = IpBlbSetAttr(BLOB_OUTLINECOLOR, 1)
	ret = IpBlbUpdate(0)
	'This next line exports the data to Excel
	ret = IpBlbSaveData("",  S_HEADER+S_Y_AXIS+S_DDE)
	'This next line pauses the macro in order to check the counted objects
	ret = IpMacroStop("Check to see if the object count looks
	appropriate",0)
	ret = IpBlbDelete()
	ret = IpSegPreview(0)
	ret = IpSegShow(1)
	ret = IpSegSetAttr(SETCURSEL, 0)
	ret = IpSegSetAttr(CHANNEL, 0)
	'These next lines redefine the range of intensities for Red, Green, and
	Blue, in order to select for regions of fatty marrow pockets
	ret = IpSegSetRange(0, 0, 255)
	ret = IpSegSetAttr(CHANNEL, 1)
	ret = IpSegSetRange(1, 120, 255)
	ret = IpSegSetAttr(CHANNEL, 2)
	ret = IpSegSetRange(2, 0, 255)
	ret = IpSegShow(0)
	ret = IpBlbUpdate(1)
	ret = IpBlbSetFilterRange(BLBM_AREA, 267.8060303, 89268.67969)
	ret = IpBlbSetAttr(BLOB_MEASUREOBJECTS, 1)
	ret = IpBlbSetAttr(BLOB_FILTEROBJECTS, 1)
	ret = IpBlbSetAttr(BLOB_CLEANBORDER, 1)
	ret = IpBlbSetAttr(BLOB_FILLHOLES, 0)
	ret = IpBlbSetAttr(BLOB_LABELMODE, 0)
	ret = IpBlbSetAttr(BLOB_OUTLINEMODE, 1)
	ret = IpBlbCount()
	ret = IpBlbSetAttr(BLOB_OUTLINECOLOR, 1)
	ret = IpBlbUpdate(0)
	ret = IpBlbSaveData("",  S_HEADER+S_Y_AXIS+S_DDE)
	ret = IpMacroStop("Check to see if the object count looks
	appropriate",0)
	ret = IpBlbDelete()
	ret = IpBlbShow(0)
End Sub
```

Vita

Ryan Michael Porter was born in Hardinsburg, KY during the summer of 1977. He spent the greater part of his as a resident of Whitesville, KY and he attended Daviess County High School in nearby Owensboro. It was here that his interest in the sciences was fostered. In the fall of 1995, Ryan enrolled at the University of Kentucky in Lexington and decided to major in Chemical Engineering. During his time at UK, Ryan participated in the Cooperative Education Program and worked three semesters with the process engineers at Protein Technologies International's Louisville facility. He was also an active member of the local chapter of AIChE, a participant in the University Men's Chorus, and a university-employed tutor (mathematics). In May of 2000, Ryan graduated Summa Cum Laude with a Bachelor of Science in Chemical Engineering (with a minor in Chemistry).

In the fall of 2000, Ryan enrolled at the Virginia Polytechnic Institute and State University in order to pursue a Master of Science in Chemical Engineering. He focused his elective coursework on molecular biology and biomedical engineering. While completing courses and research, Ryan also worked as a teaching assistant for the department of chemical engineering. He assisted Dr. Kim Forsten Williams and Dr. Aaron Goldstein during four semesters of ChE 2124, chemical engineering simulations, which is a programming-intensive course (primarily MATLAB). Ryan also served as an instructor for one summer session of Unit Operations Laboratory under Dr. Steve York. In May of 2002, Ryan received an award for outstanding presentation (M.S. research) at the first annual student research symposium co-sponsored by the Virginia Tech Center for Biomedical Engineering and the Wake Forest School of Medicine.

Following the completion of his M.S. research, Ryan plans to pursue a Doctor of Philosophy in Chemical Engineering. His research interests include multiple aspects of biomedical engineering, particularly those aspects pertaining to the musculoskeletal system.