OSTEOGENIC DIFFERENTIATION FROM MOUSE EMBRYONIC STEM CELLS AND THE ROLE OF CALRETICULIN

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science in the Graduate Department of Laboratory Medicine and Pathobiology, in the University of Toronto

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Abstract

Calreticulin, an endoplasmic reticulum (ER)-resident protein, is a calcium buffering chaperone. In this study, with an optimized differentiation protocol from mouse R1 ES cells, we demonstrate a novel role of calreticulin in osteogenic commitment and differentiation. To enhance the efficacy of the method, we manipulated cell density and examined the addition of retinoic acid, dexamethasone and peroxisome proliferator-activated receptor γ. The regimen consisting of seeding 250 cells per embryoid body, with the addition of RA (from day 3 to 5) and Dex (from day 10 to 21) gave the most efficacious output. Using this optimized protocol, we investigated the potential involvement of calreticulin in osteogenesis. Calreticulin knock-out cells displayed impaired osteogenesis compared to wild-type cells. In particular, the nuclear translocation of the runt-domain related transcription factor 2 and Osterix, were impaired in the absence of calreticulin. The stimulatory effect of calreticulin on osteogenesis was mediated by its calcium buffering function.
Acknowledgements

First, I would like to thank my family and friends for their support and encouragement.

I am grateful to my supervisor, Dr. Michal Opas, who has given me my start in research. I really appreciate your knowledge, patience, wisdom, and your one of a kind personality.

I would like to thank my committee members, Dr. Craig Simmons and Dr. Jane Mitchell, for their invaluable expert advice and scientific input in the past two years. I am also grateful to the Graduate coordinator, Dr. Harry Elsholtz, who had helped me tremendously throughout my undergraduate and graduate studies in LMP.

I would like to thank former and current members of the Opas lab. Ewa, Layla, Peter, Carlos, thank you for all that you have taught me and for your support. Without you guys, this journey would not have been possible.

Finally, I would like to thank everyone else who has helped me along the way.
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Abbreviations

AA - ascorbic acid
ALP - alkaline phosphatase
ATF4 – activating transcription factor 4
BMP-2 - bone morphogenetic protein-2
BSP - bone sialoprotein
B-Gly - β-Glycerophosphate
Ca\(^{2+}\) - calcium
C/EBP\(\alpha\) - CCAAT-enhancer-binding protein alpha
Col 1 - type 1 collagen
Col 10 - type 10 collagen
Dex - dexamethasone
EB - embryoid body
EGTA - ethylene glycol tetraacetic acid
ER - endoplasmic reticulum
ES - embryonic stem
FITC - fluorescein isothiocyanate-conjugated
GFP - Green Fluorescent protein
InsP\(_3\)R - inositol 1,4,5-triphosphate receptor
KO - knock-out
LIF - leukemia inhibitory factor
MEF2C - myocyte-specific enhancer factor 2C
MSX2 – msh homeobox 2
NFAT - nuclear factor of activated T-cells
OCN - osteocalcin
OSX - osterix
PBS - phosphate buffered saline
PPAR\(\gamma\) - peroxisome proliferator-activated receptor
PI - propidium iodide
RA - retinoic acid
Runx2 - runt-related transcription factor 2
SERCA - sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase
SOX9 - sex determining region Y-box 9
STAT1 - signal transducer and activator of transcription 1!
INTRODUCTION

Skeletal functions and development

The skeleton of mammals is maintained through the bone-forming activities of osteoblasts and bone-resorbing activities of osteoclasts (Fig. 1A). Proper maintenance of the mature skeleton is crucial for its functions such as mechanical support, muscle attachment, storage of calcium (Ca$^{2+}$) and phosphorus, as well as regulation of serum phosphorus levels (Long, 2012). Osteoblasts are cells specialized in the production of extracellular matrix (mainly collagen type 1 (Col 1)) and the mineralization process in bone (Aubin, 2001).

During vertebrate embryogenesis, bones are formed from two different processes, endochondral ossification and intramembranous ossification (Fig. 1B). These two mechanisms are responsible for forming different types of bones in the vertebrate skeleton. For instance, bones in the craniofacial regions and the clavicles are produced directly from osteoblasts via the process of intramembranous ossification, while the rest of the skeleton such as the axial components and the limbs utilizes a cartilage framework formed by chondrocytes which is replaced by bone and bone marrow by the process of endochondral ossification (Olsen et al. 2000). In this process, the mesenchymal progenitors condense and differentiate into chondrocytes and perichondrial cells. These chondrocytes eventually become hypertrophic and secrete factors to initiate the differentiation of perichondrial cells into osteoblasts (Long, 2012). Furthermore, different parts of the vertebrate skeleton have different embryonic origins. Cranial neural crest cells give rise to the craniofacial skeleton, the limb bones are formed...
Figure 1. **Skeletal maintenance and development.** **A.** The bone formation activities of osteoblasts and bone resorbing activities of osteoclasts are crucial for the proper maintenance of the skeleton. **B.** During embryogenesis, bone is formed from either intramembranous or endochondral ossification.
Figure 1

from cells from the lateral plate mesoderm, whereas somites (paraxial mesoderm) give rise to the axial skeleton (Olsen et al. 2000).

**Stages of osteogenic differentiation**

Osteogenic lineage cells are a population of cells that include mesenchymal progenitors, preosteoblasts, osteoblasts, osteocytes, and bone-lining cells. The identities of cells at different stages of differentiation are not well defined. Preosteoblasts are heterogeneous since they comprise all cells differentiating from progenitors to mature osteoblasts; they express the transcription factor the runt-related transcription factor 2 (Runx2), or both Runx2 and Osterix (OSX) at a later stage (Long, 2012). Osteoblasts express unique combination of extracellular proteins such as osteocalcin (OCN), alkaline phosphatase (ALP), and Col 1 (Long, 2012). Col 1, which is abundant in the extracellular matrix of bone, is first synthesized by osteoblasts; Ca$^{2+}$ phosphate is then deposited on this matrix in the form of hydroxyapatite. Some populations of osteoblasts become osteocytes when they are trapped in the bone matrix that they produced. Others can undergo apoptosis or become inactive bone-lining cells (Bonewald, 2011). Osteocytes are important in responding to mechanical and hormonal signals, playing major roles in bone remodeling (Bellido et al. 2005; Chen et al. 2010).

**Transcription factors in skeletogenesis**

Several transcription factors are required at different stages during osteogenic differentiation. All osteoblast precursors express msh homeobox 2 (MSX2), TWIST, and sex determining region Y-box 9 (SOX9). Since MSX2 and
TWIST are osteogenic repressors, their expression must be downregulated in osteoblast precursors for osteogenesis to proceed. The role of SOX9 in osteogenesis is not well understood. Conditional knock-out (KO) of SOX9 in the limb bud mesenchyme resulted in abolition of formation of both chondrocytes and osteoblasts (Akiyama et al. 2002). In contrast, the absence of SOX9 in the neural crest cells caused cells that would normally differentiate into chondrocytes to express osteoblastic markers instead (Long, 2012; Mori-Akiyama et al. 2003). These data may be due to the fact that SOX9 is required for both chondrogenesis and osteogenesis in endochondral ossification; while during intramembranous ossification, SOX9 is a negative regulator of Runx2 and OSX in the bipotential osteochondral progenitor cells. Runx2 is indispensable for osteoblastic differentiation for both endochondral and intramembranous ossification. Runx2-null mice were found to have an abnormal cartilaginous skeleton. In these mice, osteoblast differentiation was arrested at early stages (Long, 2012). Haploinsufficiency of Runx2 in mice or in humans resulted in hypoplastic clavicles and delayed closure of the fontanelles (which are the spaces between cranial bones held together by fibrous tissues), both phenotypes are characteristic of cleidocranial dysplasia in humans (Mundlos et al. 1997; Otto et al. 1997). In the process of endochondral ossification, higher Runx2 expression induces chondrocyte hypertrophy and osteoblast differentiation. Furthermore, Runx2 expression is also upregulated at early embryonic development in the osteo-chondroprogenitor cell during mesenchymal condensation (Ducy et al. 1997; Otto et al. 1997).
OSX is another transcription factor required for the regulation of osteoblast differentiation and bone formation. OSX-deficient mice displayed abnormal bone formation, suggesting a major role of OSX in osteoblast differentiation (Long, 2012). Interestingly, OSX-null osteoblast precursors still express Runx2, suggesting OSX acts downstream of Runx2 (Long, 2012). Activating transcription factor 4 (ATF4) is required in more mature osteogenic lineage cells. It directly regulates the expression of OCN. It is also promotes amino acid import for protein synthesis by osteoblasts (ref 76 long)

**Embryonic stem (ES) cells**

ES cells are primitive cells with the potential to give rise to all types of cells in the body. Thus, they have tremendous potential in clinical applications and they have been extensively studied in the past few decades. The first isolation of mouse ES cells was reported 25 years ago by two independent groups (Evans and Kaufman, 1981; Martin, 1981), which has revolutionized studies in genetics, developmental biology, and regenerative medicine. ES cells are derived from the inner cell mass of pre-implanted blastocysts. They have the potential of self-renewal in vitro and they can also differentiate under certain conditions and give rise to cells of all three germ layers (endoderm, mesoderm, and ectoderm). These ES cells are commonly grown and maintained on a feeder layer of division-incompetent mouse fibroblasts, which are known to be important to maintain ES cells in their undifferentiated state (Khillan and Chen, 2010). To further prevent the spontaneous in vitro differentiation of ES cells, the addition of a soluble factor, leukemia inhibitory factor (LIF) is required. Subsequent LIF
withdrawal and growth in suspension leads to embryoid body (EB) formation and differentiation (Niwa et al. 2009).

**EBs: an in vitro model of ES cell differentiation**

EBs, which are three-dimensional aggregates of stem cells, are a unique in vitro model that mimics the development of embryos during early gastrulation. They represent a unique tool to investigate developmental processes and the generation of the three germ layers (endoderm, ectoderm and mesoderm) (Boheler et al. 2005). The endodermal layer can give rise to adult tissues such as the pancreas, gastrointestinal organs, and the lungs; the ectodermal layer gives rise to skin, connective tissue of the head and face, neurons, etc. The mesenchymal progenitor cell can differentiate and commit to several different cell fates such as adipocytes, cardiomyocytes, chondrocytes, and osteoblasts (Long, 2012).

Several methods that are widely used to generate the EB include: liquid suspension culture in bacteriological dishes, culture in methylcellulose semisolid media, hanging drops, suspension culture in low-adherence vessels, and spinner flask/bioreactor techniques (Kurosawa, 2007). Suspension culture in bacterial-grade dishes relies on the hydrophobicity of the nontreated polystyrene dish to prevent the attachment of the seeded ES cells (Doetschman et al. 1985). The ES cells thus form aggregates of EBs in the absence of shaking. This method of EB induction has been utilized to produce a variety of mature cell types such as neural progenitors (Plachta et al. 2004), hepatic cells (Jones et al. 2002), cardiomyocytes (Klug et al. 1996), and germ cells (Toyooka et al. 2008) from
murine ES cells. A disadvantage of this method is that because ES cells form aggregates spontaneously, the number of cells in each EB would vary. Thus, the size and shape of the EBs would be heterogeneous (Wartenberg et al. 1998). This is problematic since cell number/density, as illustrated later in Results, is an important determinant of the efficacy of the differentiation protocol. It can further affect the lineage commitment of progenitor cells.

In addition to bacterial-grade dishes, there are also other suspension culture methods using low-adherence surfaces. For instance, dishes coated with proteoglycan (Shinji et al. 1988) or uncoated dish with positively charged surfaces are commonly used to form spherical aggregates of rat hepatocytes (Kurosawa, 2007). Round-bottomed, 96-well plates with low-adherence surfaces are also commonly used to generate EBs of uniform initial seeding numbers. These plates are first coated with reagents such as a commercially available nonionic detergent (F-127 from Sigma) or a phospholipid polymer, 2-methylacryloxyethyl phosphorylcholine (MPC) that will prevent cell adhesion to the well surfaces (Kurosawa, 2007).

Culturing in methylcellulose matrix has been widely used for hematopoietic differentiation of ES cells (Keller et al. 1993; Wiles and Keller, 1991). Since the media is semisolid, the seeded ES cells remain isolated from each other, and these single ES cells will develop into aggregates of EBs. However, handling of semisolid solution by pipettes can be quite difficult and the methylcellulose matrix can disturb the distribution of soluble factors added during differentiation (Kurosawa, 2007).
The hanging drop culture has been widely used for ES cell differentiation to a variety of cell types such as neuronal cells, cardiomyocytes, gametes and osteoblasts (Keller, 1995). Twenty to 30 μL drops containing ES cells are pipetted on the lid of Petri dishes. The lids are then inverted and placed over the bottom of the Petri dish. Gravity causes ES cells to aggregate at the bottom of the hanging drop and form EBs. The number of ES cells per EB can be controlled by varying the number of ES cells in the initial cell suspension.

The EB model has been used previously to elucidate important factors and mechanisms underlining the differentiation of mouse ES cells to osteoblasts (Kawaguchi et al. 2005; Zur Nieden et al. 2003). Several studies used the formation of EBs to generate osteoblasts in vitro within 3-4 weeks in the presence of osteogenic factors, while others have also succeeded in generating osteoblasts directly from ES cells in 10 days, without the formation of EBs (Duplomb et al. 2007b; Hwang et al. 2006).

In all of these studies, the formation of osteoblasts and bone tissue have been characterized by the capacity of the differentiated cells to mineralize and to express elevated mRNA levels of osteoblastic marker genes such as OCN, Runx2, ALP, OSX and bone sialoprotein (BSP). BSP and OCN are both extracellular matrix proteins. They are markers for late osteoblastic differentiation and are required for bone mineralization (Malaval et al. 2008; Martin, 1981).

There are several protocols in the literature attempting to enhance osteogenic differentiation from mouse ES cells. The addition of soluble factors such as β-glycerophosphate (B-Gly), ascorbic acid (AA), dexamethasone (Dex),
retinoic acid (RA) and even peroxisome proliferator-activated receptor γ (PPARγ) inhibitors have been shown to increase the efficacy of osteogenesis in vitro from ES cells (Akiyama et al. 2002; Buttery et al. 2001; Phillips et al. 2003; Yamashita et al. 2006). Table 1 provides a summary of the different factors which have been studied to enhance osteogenic differentiation from ES cells.

Table 1. A summary of the studies investigated the effects of different factors promoting osteogenesis from ES cells.

<table>
<thead>
<tr>
<th>References</th>
<th>Cell Line Used</th>
<th>Factors Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Kuske et al. 2011)</td>
<td>D3 (mouse)</td>
<td>B-Gly, AA, BMP-2, vitamin D₃.</td>
</tr>
<tr>
<td>(Kawaguchi et al. 2005)</td>
<td>CGR8, E14Tg2a (both mouse)</td>
<td>B-Gly, RA, AA, RA, Dex.</td>
</tr>
<tr>
<td>(Buttery et al. 2001)</td>
<td>CEE (mouse)</td>
<td>B-Gly, AA, RA, Dex (both early and late addition).</td>
</tr>
<tr>
<td>(Bourne et al. 2004)</td>
<td>CEE (mouse)</td>
<td>B-Gly, AA, Dex.</td>
</tr>
<tr>
<td>(Woll and Bronson, 2006)</td>
<td>HM1 (mouse)</td>
<td>B-Gly, AA</td>
</tr>
<tr>
<td>(Yamashita et al. 2006)</td>
<td>SV6 (mouse)</td>
<td>PPARγ siRNA.</td>
</tr>
<tr>
<td>(Rose et al. 2012)</td>
<td>D3 (mouse)</td>
<td>Basic fibroblast growth factor.</td>
</tr>
</tbody>
</table>

These studies have either used mouse or monkey ES cells. Abbreviations: B-Gly: beta-glycerophosphate; AA: ascorbic acid; RA: retinoic acid; Dex: dexamethasone, PPARγ: peroxisome proliferator-activated receptor-gamma; BMP-2: bone morphogenetic protein-2.
Importantly, it also has been demonstrated that the initial cell number comprising the EBs and/or their size affects the end product of differentiation (McBeath et al. 2004; Park et al. 2007). This parameter has not been optimized in osteogenic differentiation protocols. Thus, before further studies of molecular mechanisms controlling osteogenesis, it is necessary to optimize culture conditions and to use the most efficacious differentiation protocol.

**Calreticulin**

The endoplasmic reticulum (ER) has crucial roles in many cellular processes such as Ca\(^{2+}\) storage, lipid and protein synthesis, protein folding and post-translational modifications. These diverse functions are carried out by many proteins that reside in the ER lumen. Calreticulin is a 46 kDa ER luminal Ca\(^{2+}\)-binding protein and molecular chaperone. It has numerous important roles in biological processes such as apoptosis, immunity, cancer, angiogenesis, wound healing, cardiac development, stem cell differentiation and central nervous system development (Michalak et al. 2009). Calreticulin was first isolated in 1974 by Ostwald and MacLennan (1974). Genes encoding calreticulin have been isolated from several vertebrates, invertebrates, and higher plants (Michalak et al. 1999). There is no calreticulin gene in the genomes of yeast and prokaryotes. The human gene for calreticulin is located on chromosome 19 at locus p13.3-13.2 and in the mouse on chromosome 8 (Gelebart et al. 2005). The nucleotide sequences of the mouse and human gene displays greater than 70% identity, suggesting a strong evolutionary conservation of the gene (Gelebart et al. 2005).
A 2002 study identified a second isoform of calreticulin (Persson et al. 2002); however, the physiological function of this newly identified protein is unclear, but it is abundantly expressed in the testes.

The presence of a N-terminal cleavable signal sequence directs calreticulin to the ER, and it also contains an ER KDEL (Lys-Asp-Glu-Leu) retention/retrieval signal. Calreticulin, together with calnexin and ERp57 (ER protein of 57 kDa), is involved in the chaperoning of nascent polypeptides that traverse through the ER (Hebert and Molinari, 2007). It has three domains, the N domain, P domain and the C domain (Fig. 2). Structural studies revealed that the N-domain is a globular structure comprised of eight anti-parallel β-strands (Michalak et al. 1999). The highly conserved N-domain has been shown to interact with the DNA-binding domain of the glucocorticoid receptor in vitro (Burns et al. 1994), and with protein disulphide-isomerase (PDI) and ER protein 57 (ERp57) (Baksh et al. 1995; Corbett et al. 1999). Interestingly, these protein-protein interactions have been shown to be dependent on Ca\(^{2+}\) binding to the C-domain of calreticulin (Corbett et al. 1999). The proline-rich P-domain of calreticulin is critical for the high-affinity Ca\(^{2+}\) binding and lectin-like chaperone activity of calreticulin (Tjoelker et al. 1994; Vassilakos et al. 1998). The C-domain binds to over 25 mol of Ca\(^{2+}\)/mol protein (Baksh and Michalak, 1991) and binds to blood-clotting factors (Kuwabara et al. 1995).

Molecular chaperones prevent the aggregation of partially folded proteins, and increase the amount of correctly folded proteins. It has been widely documented that calreticulin functions as a lectin-like molecular chaperone
Figure 2. The structural and functional domains of calreticulin. Calreticulin has three domains: the N, P and C domains. Its N and P domains are responsible for its chaperoning function. The C domain carries out its high capacity low affinity Ca\textsuperscript{2+}-buffering activity.
(Michalak et al. 1999). Its chaperone activity is carried out by the N and P domains; while its high capacity Ca$^{2+}$ binding activity is found in its C domain. Calreticulin and calnexin, an integral ER membrane protein, are both highly versatile proteins that are involved in “quality control” during the synthesis of a variety of molecules (Coe and Michalak, 2009) such as ion channels, surface receptors, and transporters (Michalak et al. 1999; Michalak et al. 2009).

**Calreticulin and Ca$^{2+}$ buffering function**

Ca$^{2+}$ is a universal signalling molecule that influences many developmental and cellular processes such as muscle contraction, cell proliferation and cell death, neurotransmitter release, etc. The majority of the intracellular Ca$^{2+}$ is stored in the ER lumen. Extracellular Ca$^{2+}$ level is 2mM, total ER Ca$^{2+}$ concentration is up to 1mM, free ER Ca$^{2+}$ concentration is approximately 200 µM, and finally, the free cytoplasmic Ca$^{2+}$ concentration is only 100 nM (Michalak et al. 2009). Ca$^{2+}$ release from the ER via inositol 1,4,5-trisphosphate receptor (InsP$_3$R) and ER Ca$^{2+}$ uptake by the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) are crucial in maintaining ER Ca$^{2+}$ homeostasis and signalling. The C-domain of calreticulin contains many negatively charged residues that are responsible for the Ca$^{2+}$-buffering function of the protein (Michalak et al. 2009). It binds over 50% of ER luminal Ca$^{2+}$ with high capacity (25 mol of Ca$^{2+}$ per mol of protein) and low affinity ($K_d=2$ mM) (Nakamura et al. 2001). Although the P-domain of calreticulin also binds Ca$^{2+}$, it does so with high affinity ($K_d=1$ µM), but low capacity (1 mol of Ca$^{2+}$ per mol of protein) (Baksh and Michalak, 1991). Considering the high concentration of Ca$^{2+}$ within the lumen of
the ER, the high affinity Ca$^{2+}$-binding site of calreticulin has been predicted to be permanently occupied by Ca$^{2+}$ and probably serves a structural role only. Consequently, the Ca$^{2+}$ buffering function of calreticulin is mainly carried out by its C-domain. Calreticulin is responsible for binding over 50% of the ER free Ca$^{2+}$ (Michalak et al. 2009). This critical role of calreticulin as a regulator of Ca$^{2+}$ homeostasis is further demonstrated in loss-of-function and gain-of-function experiments. Upregulation of calreticulin lead to increased Ca$^{2+}$ capacity of the ER, increased free Ca$^{2+}$ concentration in the ER lumen, and a larger pool of releasable Ca$^{2+}$ from the ER, whereas calreticulin deficiency leads to reduced Ca$^{2+}$ storage capacity in the ER and impaired Ca$^{2+}$ release from the ER (Arnaudeau et al. 2002; Mery et al. 1996).

**Calreticulin and embryonic development**

Since calreticulin is involved in diverse basic cellular functions, it was expected that calreticulin-null mice would not be viable due to early embryonic lethal phenotype. In fact, calreticulin deficiency is embryonic lethal between day 12.5 and 14.5 due to abnormal cardiac development. Experiments using calreticulin-null mice and ES cells showed that the absence of calreticulin lead to impaired myofibrillogenesis (Li et al. 2002; Lozyk et al. 2006) and deficient intercalated disc formation in the hearts. Further investigations revealed that calreticulin affects transcription factors involved in cardiac development such as the Nuclear factor of activated T-cells (NFAT) and Myocyte-specific enhancer factor 2C (MEF2C) via its Ca$^{2+}$ buffering activity (Michalak et al. 2009). In addition to heart defects, calreticulin-null embryos also had disrupted cranial
neural tube closure and displayed exencephaly (Rauch et al. 2000). This is not surprising since calreticulin is abundant in the developing brain (Mesaeli et al. 1999). Further studies demonstrated that slower cell migration and decreased cell adhesion caused by calreticulin deficiency could have led to this phenotype (Rauch et al. 2000). The failure of the ventral body wall closure was also observed in the calreticulin-deficient embryos. The mechanism by which this is regulated by calreticulin is unclear; however, it is highly plausible that calreticulin’s effects in cell migration and attachment may have a role (Rauch et al. 2000).

**Calreticulin affects gene expression and cell differentiation**

During cardiomyogenic differentiation, the expression of calreticulin in the developing heart is high and then it declines (Mesaeli et al. 1999). Cardiac transcription factors Nkx2.5 and MEF2C activate the calreticulin gene (Guo et al. 2001). Nkx2.5 then combines with GATA-4 transcription factor to further enhance their activity (Nemer and Nemer, 2001). Furthermore, the combined activities of the GATA-4 and NFAT transcription factors are also critical for cardiomyogenesis (Nemer and Nemer, 2001). NFAT must be dephosphorylated by calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase (Crabtree, 2001) in order to translocate to the nucleus. Calreticulin was found to regulate the transcriptional activity of the GATA-4/NFAT complex by affecting calcineurin activity through the control of ER Ca^{2+} release (Lynch and Michalak, 2003). Thus, the absence of calreticulin leads to inactivation of the critical cardiac transcription factors, leading to impaired cardiomyogenesis.
In addition to its involvement in cardiomyogenic development, it was discovered that calreticulin also affects mesenchymal stem cell commitment towards the adipogenic lineage. Calreticulin was found to act as a Ca\(^{2+}\)-dependent molecular switch that inhibits the commitment of ES cells to the adipogenic lineage. PPAR\(\gamma_2\) and CCAAT-enhancer-binding protein alpha (C/EBP\(\alpha\)) are both crucial transcription factors during adipocyte development (Rosen and Spiegelman, 2000). Binding of PPAR\(\gamma_2\) to the calreticulin gene promoter enhances the expression of calreticulin. This increase in expression of calreticulin downregulates the expression and transcriptional activation of PPAR\(\gamma_2\) and C/EBP\(\alpha\), forming a negative feedback loop (Szabo et al. 2008).

**Calreticulin and Skeletogenesis**

There are limited data available on calreticulin expression or function in osteogenic cells *in vivo* or *in vitro*. It is well known in the literature that calcineurin, a serine-theronine phosphatase that is regulated by calreticulin (Guo et al. 2002; Lynch et al. 2005), plays an important role in chondrogenesis (Reinhold et al. 2004) and osteogenesis (Sun et al. 2005). Hence, it is highly plausible that calreticulin may be affecting osteocyte and chondrocyte differentiation via the calcineurin pathway.

Previous studies have shown calreticulin’s critical roles in adipogenesis and cardiomyogenesis (Lynch et al. 2005; Szabo et al. 2008). However, its function in osteogenesis remains largely unknown; thus, the role of calreticulin in osteogenic differentiation from mouse ES cells is examined in this study.
HYPOTHESIS

Calreticulin has a critical role in the osteogenic differentiation from mouse ES cells.

Aims:

1) To optimize culture conditions for osteogenic differentiation from mouse ES cells
2) To determine whether calreticulin has a role in osteogenic differentiation

METHODS AND MATERIALS

Cell Culture

Osteogenic lineage differentiation was performed from G45 calreticulin KO ES cells, CGR8 ES cells (both cell lines derived from J1 129/SV mice and were kindly provided by Dr. Marek Michalak, Department of Biochemistry of University of Alberta) or R1 mouse ES cells (derived from J1 129/Sv mice, purchased from Dr. Janet Rossant, Mount Sinai Hospital, Toronto, Canada) (Nagy et al. 1993) by the hanging drop method. The ES cells were maintained on mitomycin C-treated mouse embryonic fibroblast feeder cells on gelatin-coated plates. At passage 2, cells were trypsinized and collected for hanging drops. To test the effect of cell number and/or EB size on the osteogenic lineage differentiation, varying numbers of ES cells per 25 µL drops were placed on the lids of tissue culture dishes for 3 days (Fig. 3). The medium consisted of: high glucose Dulbecco modified Eagle’s medium with sodium pyruvate and L-glutamine (Multicell,
Wisent), 20% Fetal Bovine Serum (Multicell, Wisent), nonessential amino acids, and β-mercaptoethanol. After 3 days in hanging drop, these ES cells aggregated and formed EBs and then were floated in differentiation medium for 2 days. On day 5, the EBs were then plated in tissue culture dishes coated with 0.1% gelatin (in phosphate-buffered saline [PBS]) in differentiation medium with 50 µg/mL AA and 10 mM B-Gly for commitment to the osteogenic lineage. The medium was changed every 2 days for the entire 21 days of differentiation.

**Treatment with putative osteogenic factors**

Dex, RA and PPARγ inhibitor (GW9662) were selected over other soluble factors because they were more frequently utilized in the literature. Although bone morphogenetic protein 2 (BMP2) was also commonly used, it has also been reported to induce chondrogenic differentiation. Another frequently used factor is vitamin D. It was not examined in this study since it has been shown that Dex can substitute its pro-osteogenic effects in the culture. The effects of Dex, RA, and PPARγ inhibition on osteogenic differentiation from ES cells were examined in six different protocols: A) Control: only AA and B-Gly addition; B) 0.1 µM Dex (Sigma-Aldrich) early addition, added at day 6 and kept throughout differentiation; C) 0.1 µM Dex late addition, added at day 10 and kept throughout differentiation; D) PPARγ inhibitor: 1 µM GW9662 (Sigma-Aldrich) is added at day 3 and kept throughout differentiation; 0.1 µM Dex is added at day 10 to further enhance mineralization. E) 0.1 µM RA (Sigma-Aldrich) added during the floating stage (from day 3 and kept until day 5), media was changed every day; F) RA added from day 3 and kept until day 5 + Dex early addition (added at day 6
Figure 3. The effect of cell number on osteogenic differentiation from mouse ES cells. The hanging drop method was used to differentiate mouse ES cells into the osteogenic lineage. Aggregates of stem cells form embryoid bodies or EBs in the first 2 days. To test the effect of cell number and/or EB size on the osteogenic lineage differentiation, varying numbers of ES cells per 25 µL drops were placed on the lids of tissue culture dishes. These EBs then go into the “floating stage” in which they grow in suspension for another 2 days. On day 5, the EBs then are attached on gelatin-coated dishes until day 21. B-Gly and AA are added into the culture media starting at day 6 and kept throughout differentiation.
Figure 3

Mouse embryonic stem cells

HD
250 cells/25 μL
500 cells/25 μL
1000 cells/25 μL

F
1000
500
250

EBs attached osteogenic factors – β glycerophosphate + Ascorbic Acid

HD: Hanging drops.
F: Floating.
EBs: Embryoid bodies.
RA: Retinoic acid.
Dex: Dexamethasone.
until day 21); G) RA added from day 3 until day 5 + late addition of Dex (added at day 10 until day 21). Protocols B) to G) also contain B-Gly and AA. A schematic diagram representing the above protocols and the timeline of differentiation can be found in Fig. 4.

**Ca\(^{2+}\) Manipulation Studies**

The ionomycin and 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (abbreviated BAPTA-AM) treatments were performed as previously described by Szabo *et al.* (2008). To chelate cytoplasmic Ca\(^{2+}\), floating EBs were incubated with 500 nM BAPTA-AM for 30 min each day from days 3 to 5. To increase cytosolic Ca\(^{2+}\), 1 \(\mu\)M ionomycin was added to the media for 2 hr each day from days 3 to 5.

**Mineralization Assays**

von Kossa staining is a routine method for detecting Ca\(^{2+}\) deposits in culture. Osteoblasts are known to produce a matrix of osteoid, which is mainly composed of Col 1. Ca\(^{2+}\) and phosphate deposition takes place later by osteoblasts, resulting in the formation of hydroxyapatite crystals (Field *et al.* 1974). von Kossa staining is based on the visualization of reduced silver ions that replaced Ca\(^{2+}\) ions which were bound to phosphate in tissue deposits (Rungby *et al.* 1993). von Kossa staining was performed on differentiated day 21 nodules to detect mineralization. Before staining, cells were washed three times with PBS, fixed in 10% neutral formalin buffer for 2 hr, and then washed 3 times with distilled water. Nodules were then stained with 2.5% silver nitrate solution for 30 min. Finally, they were washed 3 times with distilled water and were examined.
Figure 4. The effect of different soluble factors on osteogenic differentiation. The hanging drop method was used to study the effects of different soluble factors on osteogenesis from mouse ES cells. The different combinations of osteogenic factors tested are: **A.** control with AA and B-Gly addition; **B.** 0.1 µM Dex Early, added at day 6 and kept throughout differentiation; **C.** 0.1 µM Dex Late, added at day 10. **D.** 1 µM PPARγ inhibitor GW9662 is added at day 3 and kept throughout differentiation; 0.1 µM Dex is added at day 10 to further enhance mineralization. **E.** 0.1 µM RA added from day 3 and kept until day 5; **F.** 0.1 µM RA added from day 3 and kept until day 5 + Dex Early (added at day 6 until day 21.) **G.** 1 µM RA added from day 3 until day 5 + Dex Late (added at day 10 until day 21).
Figure 4

Mouse embryonic stem cells

HD: hanging drops
F: floating
EBs: embryoid bodies
RA: retinoic acid
Dex: dexamethasone

EBs attached
osteogenic factors – β-glycerophosphate + ascorbic acid

Days

A

B

C

GW9662 1 μM

D

RA 0.1 μM

E

RA 0.1 μM

F

RA 0.1 μM

G

RA 0.1 μM

Dex 0.1 μM

Dex 0.1 μM

Dex 0.1 μM

Dex 0.1 μM

Dex 0.1 μM

Dex 0.1 μM
for the presence of mineralization. For a quantitative measure of the amount of Ca\(^{2+}\) in the mineralized nodules, the Arsenazo III method (a Ca\(^{2+}\)-sensitive dye) was used (Zayzafoon et al. 2004). Briefly, the differentiated bone nodules at day 21 were collected and incubated with 3 M HCl at 80°C for 1 to 2 hr. The resulting suspension was neutralized to pH 5 with 3 M Trizma base, and mixed with Arsenazo III (100 µM), after 5 min incubation the intensity of resulting colour were measured at 595 nm. The total Ca\(^{2+}\) amount was calculated by dividing it by the total protein determined from a duplicate well. The resulting values were expressed as mmol of Ca\(^{2+}\)/µg of total protein.

**Alcian blue staining**

Differentiated day 21 nodules were rinsed three times with PBS, and fixed in 10% neutral formalin buffer for at least 15 min or overnight. After rinsing 3 times with distilled water, nodules were left in 2 changes of 0.5 N HCl, 3 min each. Cells were then stained with 0.25% Alcian Blue overnight. Immediately following the removal of the stain, cells were rinsed with 0.5 HCl 3 times, 3 min each. Finally, cells were rinsed with slowly running tap water and then were immediately observed.

**Real-time Polymerase Chain Reaction (PCR)**

Total RNA was extracted from day 21 nodules using the Qiagen RNeasy Mini Kit according to the manufacturer’s instructions. 4 µg of RNA was reverse transcribed to cDNA using Superscript II (Invitrogen) in a total reaction volume of 48 µL. To examine the mRNA expression of osteogenic markers, cDNA was amplified using Real-time PCR. Real-time PCR analysis was performed in Bio-
Rad’s CFX384 Touch™ detection system. The accumulation of PCR products was monitored by measuring the fluorescence caused by the binding of SYBR Green (Applied Biosystems) to double-stranded DNA. The cDNA levels were normalized using ribosomal protein L32 (a housekeeping gene). The primer sequences that were used are as follows:

for L32, forward primer 5’-CATGGCTGCCCTTCGGCCTC-3’ and reverse primer 5’-CATTCTCTTTCGCTGCGTAGCC-3’;
for Runx2, forward primer 5’-CCTCTGACTTCTGCCTCTGG-3’ and reverse primer 5’-TAAAGGTGGCTGGGTAGTG-3’;
for OSX, forward primer 5’-GAAAGGAGGCACAAAGAG-3’ and reverse primer 5’-CACCAAGGAGTAGGTGTGTT-3’;
for calreticulin, forward primer 5’-AAGTTCTACGTTGAGGAGGAG-3’ and reverse primer 5’-GTCTATTTCTGCTCAGGT-3’;
for Col 2, forward primer 5’-CACTGGTGGGTGAGAGCAG-3’ and reverse primer 5’-GGATTGTGTTGTTTCACCGGTTTC-3’;
for aggrecan, forward primer 5’-GGTGGATCTGGCATGAGAG-3’ and reverse primer 5’-GGTGCCCTTTACACGTGA-3’;
for BSP, forward primer 5’-ACCCCAAGCAGACACTTGTG-3’ and reverse primer 5’-CTTTCTGCATCTCCAGCTTCT-3’;
and for OCN, forward primer 5’-AAGTCCACACAGCAGCTG-3’ and reverse primer 5’-AGCCGAGCTGCCAGGTT-3’.
Immunofluorescence Staining

Day 21 differentiated nodules grown on coverslips were rinsed 3 times with PBS. They were fixed in 3.7% formaldehyde for 15 min. After washing (three times for 5 min each time) in PBS, the cells were permeabilized with 0.1% Triton X-100 in buffered containing 100 mM 1,4-piperazinediethanesulfonic acid, 1 mM ethylene glycol tetraacetic acid (EGTA), and 4% (wt/vol) polyethylene glycol 8000 (pH 6.9) for 2 min. Nodules were then rinsed 3 times in PBS for 5 min and they were incubated with primary antibodies (dilution of 1:50) for 30 min at room temperature. The primary antibodies used include: anti-Runx2 (Abcam) and anti-OSX (Abcam). After washing in PBS (3 times for 5 min), the cells were stained with the secondary antibody (fluorescein isothiocyanate-conjugated [FITC] donkey anti-mouse, or anti-rabbit; diluted 1:50) for 30 min at room temperature. After, nodules were washed 3 times in PBS for 5 min. Nodules were incubated with 100 ug/mL DNase-free RNase in PBS for 30 min at 37°C, followed by rinsing in PBS 3 times, 1 min each. For nuclear staining, 500 nM Propidium Iodide (PI) solution was added to cover the nodules for 5 min. After final rinsing in PBS (3 times for 1 min each), the slides were mounted in fluorescent mounting medium (Dako). A confocal fluorescence microscope (MRC 600; Bio-Rad Laboratories) equipped with a 60/1.40 planapochromatic oil immersion objective (Nikon) and krypton-argon laser was used for fluorescence imaging. To avoid bleed-through of fluorescence between channels, we imaged FITC and PI using separate, single excitation filters and single fluorochrome filter/mirror blocks: 10 nm 488 nm bandpass excitation filter and BHS block with 510 nm longpass
dichroic mirror and 515 nm long pass OG filter versus 10 nm 514 nm bandpass excitation filter and YHS block with 540 nm longpass dichroic mirror and 550 nm long pass OG filter, which prevented any spillover of fluorescent signal.

Transmission Electron Microscopy

Samples for hydroxyapatite imaging by transmission electron microscopy (TEM) were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer followed by rinsing in the same buffer. They were then fixed in 1% osmium tetroxide in buffer, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in Quetol-Spur resin. Sections 100 nm thick were cut on an RMC MT6000 ultramicrotome, stained with uranyl acetate and lead citrate and viewed in an FEI Tecnai 20 TEM.

Statistical Analysis

All statistical analyses were performed using two-way analysis of variance (ANOVA; with post hoc Bonferroni test) or independent t-test (IBM SPSS, version 21). Experiments were independently repeated at least three times, each repeated in triplicate unless otherwise stated. Values are expressed as the mean ± SD. Differences between mean values for different treatments were considered to be significant at P<0.05 and P<0.01.

RESULTS

Aim 1: To enhance the efficacy of mouse ES cell osteogenic differentiation protocol

The hanging drop protocol which had been previously utilized in our laboratory for cardiomyogenic and adipogenic differentiation was modified for
osteogenic differentiation from mouse ES cells (Papp et al. 2009; Szabo et al. 2009; Szabo et al. 2008). Mouse ES cells were placed on the lids of tissue culture dish for 3 days in differentiation media. On day 3, the formed EBs were transferred to bacteriological dishes in which they grew in suspension for 2 days. On day 5, the EBs were then plated on gelatinized tissue culture dishes. To enhance osteogenic differentiation, the medium was supplemented with 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate on day 5. The mouse cell line used initially was CGR8, a widely used ES cell line developed by Austin Smith (Dirks et al. 1995). However, this protocol did not yield a high output of osteogenic lineage cells from the CGR8 cells. von Kossa staining of differentiated day 21 nodules did not show high levels of mineralization, although PCR analysis revealed that these nodules did express Runx2, OSX, OCN, and BSP. The experiment was repeated 3 times to confirm the efficacy of the protocol. Additional experiments confirmed the low mineralization results of the initial trial. Furthermore, the expression levels of osteogenic markers were inconsistent across experiments. Thus, I employed a different ES cell line, R1 (one of the most commonly used mouse ES cell line that has been isolated by Andras Nagy (Nagy et al. 1993)) to determine whether more consistent results would arise. R1 cells had higher level of mineral deposition and expressed higher levels of osteogenic markers than CGR8 cells. Experiments using R1 cells had better reproducibility than CGR8 cells. Thus, the remainder of the current project utilized the R1 cell line instead. Before I commenced my study on the role of calreticulin in osteogenesis, I proceeded to explore different culture conditions.
with the aim to further increase the efficacy of our osteogenic differentiation protocol. The results are presented below.

**The effect of cell number on osteogenic differentiation**

The generation of the EB is a critical step in ES cell differentiation. It has been shown that the cell number forming these EBs and their size has crucial effects on the downstream product of ES cell differentiation (McBeath et al. 2004; Park et al. 2007). To determine the effects of cell number/density on osteogenesis, EBs consisting of 250, 500, or 1000 ES cells were allowed to grow under osteogenic condition using the hanging drop method (Fig. 3). Detection of osteogenic differentiation in the day 21 nodules was performed by mineralization assays and Real-time PCR for the expression of osteogenic markers. Both von Kossa staining and Arsenazo III revealed that nodules resulting from EBs of 250 cells gave the highest level of mineralization compared with the other two cell numbers (Figs. 5A and B). Furthermore, nodules resulting from EBs of 250 cells showed the highest expression level of osteogenic markers (Runx2, OSX, and OCN) compared to those derived from other cell numbers (Fig. 5C). To correct for loading errors, all mRNA expression values were normalized against the housekeeping gene L32. For all real-time PCR raw data, please see Appendix.

**The effects of Dex on osteogenesis**

Dex has been shown to have stimulatory effects on osteogenic differentiation which include increased ALP activity and mRNA expression (Ogston et al. 2002). To determine the effect of Dex on mouse ES cell differentiation 0.1 µM of Dex was added in the media either starting at day 6 or
Figure 5. The lowest cell number and/or EB size favoured osteogenic differentiation. A and B. At day 21 of differentiation, nodules derived from EBs containing either 250, 500, or 1000 cells were examined for the level of mineralization using von Kossa staining and the Arsenazo II stain. Both methods revealed that nodules derived from the initial seeding number of 250 cells resulted in the highest level of mineralization. C. Upon analysis using Real-time PCR, nodules differentiated from EBs containing 250 cells expressed the highest level of osteogenic markers (Runx2, OCN, and OSX). Conditions were performed in triplicates, data are presented as means ± SD of three independent experiments (* represents p < 0.05 and ** represents p < 0.01).
Figure 5

A

B

C

* p < 0.05 vs 250 cells
day 10 (Fig. 4). Dex addition in the media enhanced mineralization (Figs. 6A and B) and the transcription of the critical osteogenic transcription factor Runx2 was upregulated (Fig. 6C); the effect of late addition of Dex at day 10 lead to more profound results compared to early addition at day 6. Early addition of Dex gave only a 2-fold increase in osteogenic marker OCN mRNA; while there was 4-fold increase with late Dex addition (Fig. 6C). Similarly, early addition of Dex enhanced the mRNA expression of Runx2 by 12-fold compared to the control; while in the cells treated with Dex later in differentiation, there was a 22-fold increase in Runx2 mRNA expression. Fig. 6C shows that both early and late addition of Dex increased the expression of OSX compared to their control.

**The effects of RA on osteogenesis**

The effect of RA treatment during the floating stage on the commitment and differentiation of ES cells to the osteogenic lineage can be seen by enhanced mineralization (Figs. 6A and B). Furthermore, the osteogenic markers, Runx2 and OCN, also had a 33-fold and 5.5-fold increase in their mRNA expression, respectively (Fig. 6C). The expression of OSX was increased by 22.3-fold compared to the non-treated control (Fig. 6C).

**The combined effects of RA and Dex on osteogenesis**

Thus far, the treatment with either RA or Dex alone enhanced osteogenic differentiation from ES cells. When they were both added together into the media, this further enriched the ES cells commitment and differentiation to the osteogenic lineage than when the factors were added separately (Fig. 6). The addition of both RA and Dex (early addition at day 6) lead to a 46.7-fold increase
Figure 6. Mineralization assays and PCR analysis revealed that the RA and later addition of Dex treatment when combined, resulted in the highest level of osteogenic differentiation. A. von Kossa staining was used to detect mineralization in day 21 nodules treated with different combinations of osteogenic factors. Nodules were fixed in formaldehyde and then stained with 2.5% silver nitrate solution. The combination of RA and late addition of Dex gives the most mineralization compared to other groups upon von Kossa staining. B. Quantification of extracellular Ca$^{2+}$ content by Arsenazo III kit confirmed the von Kossa results. C. Real-time PCR analysis revealed that RA and later addition of Dex treatment resulted in the highest level of expression of osteogenic markers. Conditions were performed in triplicates, data are presented as means ± SD of 3 independent experiments. (Abbreviations: E: early, L: late).
Figure 6

A

B

C

Runx2

OCN

OSX

* p<0.01 vs Control
# p<0.01 vs Dex E
§ p<0.01 vs RA Dex L

* p<0.01 vs RA Dex L
# p<0.01 vs RA Dex E

* p<0.01 vs RA
# p<0.01 vs RA Dex E
§ p<0.01 vs RA
in Runx2 mRNA expression and a 7.3-fold increase in OCN mRNA expression compared to control (Fig. 6C). The mRNA expression of OSX was increased 43.3-fold in the treatment regimen of RA and late Dex addition (Fig. 6C). The combination of RA and Dex (late addition at day 10) gave the most mineralization and highest increase in osteogenic markers expression compared to all other protocols.

**The effects of PPARγ on osteogenesis**

Upon treatment of the PPARγ inhibitor, GW9662 (1 µM), mineralization was enhanced (Figs. 6A and B). The mRNA expression of Runx2 and OCN increased as well. There was a 26.9-fold increase in Runx2 mRNA transcript; a 5.6-fold increase in the OCN mRNA expression; and a 5.8-fold increase in OSX mRNA expression (Fig. 6C). The increase in the osteogenic induction by GW9662-treated nodules was not as significant compared to the addition of RA and (late) Dex.

**Confirmation of osteogenic differentiation from ES cells**

Thus far, we found that the differentiation protocol using 250 cells per EB with the addition of RA and late Dex resulted in the highest enrichment of osteogenic lineage cells compared to other treatments examined in this study. To further confirm the osteogenic lineage differentiation of the cells obtained from this protocol, we performed immunofluorescence staining and transmission electron microscopy (TEM) on the differentiated nodules. Immunofluorescence staining revealed the nuclear localization of the osteogenic transcription factors Runx2 and OSX (Fig. 7). Negative controls for the primary antibodies were
Figure 7. Immunofluorescence staining of the osteogenic markers, Runx2 and OSX. A. At day 21 of differentiation, nodules were fixed in 3.7% formaldehyde, permeabilized, and labelled for the transcription factors Runx2 or OSX. PI was used to identify the nuclei. Both Runx2 and OSX were found to be nuclear in most cells. B. Negative controls for the primary antibodies were accomplished by labelling MDCK cells with anti-Runx2 or anti-OSX antibodies. C. Negative controls for the secondary antibodies were performed by omitting primary antibodies in the staining procedure. Scale bar division represents 10 µm.
Figure 8. Transmission electron microscopy images of differentiated day 21 nodules in RA and Dex (late) protocol. The presence of hydroxyapatite deposition is detected in the extracellular matrix of the nodules. Classic modes such as bright field were used to image the texture of the deposit in different nodules. A-D shows representative pictures of the nodules examined. E shows the diffraction pattern of a selected area identifying the crystalline material as biological hydroxyapatite.
Figure 9. The most efficacious culture condition for osteogenesis from mouse R1 cells. In the different culture conditions we tested, the regimen of RA and late addition of Dex using an initial seeding number of 250 cells per EB, yielded the highest level of osteogenic induction from mouse R1 ES cells.
Figure 9

Mouse embryonic stem cells

HD: hanging drops
F: floating
EBs: embryoid bodies
RA: retinoic acid
Dex: dexamethasone

RA 0.1 μM
Dex 0.1 μM

Days

250 cells/ 25 μL

EBs attached
osteogenic factors – β-glycerophosphate + ascorbic acid
accomplished by labelling the non-osteogenic cell line, Madin-Darby canine kidney (MDCK) cells with anti-Runx2 or anti-OSX antibodies. Negative controls for the secondary antibodies were performed by omitting primary antibodies in the procedure. As shown in Figure 7, no staining was detected in all negative controls.

Although von Kossa staining is routinely used to detect the level of mineralization in osteogenic cultures, this technique is not specific since it detects the presence of Ca\(^{2+}\) which can be induced due to other biological processes such as apoptosis. Thus, it is imperative to confirm that the improved protocol (250 cells per EB, RA and late Dex treatment) does not induce nonphysiological mineral deposition using more sensitive methods such as TEM for hydroxyapatite imaging. Figure 8 shows the transmission electron micrographs illustrating the presence of hydroxyapatite crystals in the extracellular matrix which has been further confirmed with the crystalline microdiffraction pattern obtained with 1 nm electron probe (Fig. 8C). Thus, the high levels of mineral deposition in the improved protocol observed by von Kossa staining was hydroxyapatite and not any other nonphysiological forms. In summary, the optimized osteogenic differentiation protocol using initial seeding number of 250 cells per EB and RA and Dex late treatment (Fig. 9) results in the highest enrichment of osteogenic lineage cells and no nonphysiological mineralization.

**Aim 2: The role of calreticulin in osteogenic differentiation**

*Calreticulin expression during mouse ES cell osteogenic differentiation*
Real-time PCR analysis was performed in order to determine the expression profile of calreticulin throughout the 21-day osteogenic differentiation. RNA was extracted from differentiated day 21 R1 and calreticulin KO nodules; it was then reverse transcribed to cDNA, which was then analyzed using real-time PCR. Figure 10 shows the calreticulin expression profile in the 21 days of osteogenic differentiation. In calreticulin-containing R1 cells, the expression of calreticulin is low in pluripotent cells and it becomes more abundant and peaks at day 14. The expression remains relatively abundant for the rest of differentiation. Not surprisingly, in KO cells, there is no calreticulin expression throughout the differentiation process.

**Calreticulin deficiency impairs osteogenic differentiation from mouse ES cells in vitro**

To characterize the role of calreticulin in the osteogenic differentiation of mouse ES cells, we allowed the EBs from wild type (R1) and calreticulin KO ES cells to differentiate under osteogenic conditions with the additions of osteogenic factors B-Gly, AA, RA and Dex. The level of extracellular mineralization was examined at day 21, and the expression of osteogenic molecular markers, Runx2, OSX, and BSP were examined throughout differentiation. von Kossa staining for the presence of mineral deposition showed that the absence of calreticulin significantly reduced the level of mineralization in KO cells compared to their wild type counterparts (Fig. 11A). The Arsenazo III method was used to measure Ca\(^{2+}\) deposition in the matrix. It showed that wild type cells had 7-fold increase in the concentration of extracellular Ca\(^{2+}\) compared to that present in
Figure 10. The expression of calreticulin throughout osteogenic differentiation. Real-time PCR analysis revealed that calreticulin expression is low in pluripotent (plu) R1 stem cells. As differentiation commences, its mRNA expression steadily increases and peaks at day 14. Its expression remains abundant later in differentiation. In the calreticulin KO cells, no calreticulin mRNA expression is detected. Conditions were performed in triplicates, data are presented as means ± SD (* represents p < 0.05 vs day 14 value).
Figure 10

Calreticulin expression in osteogenic differentiation

* p < 0.05 vs Day 14
the KO nodules (Fig. 11B). Real-time PCR analysis revealed that the mRNA expression of the osteogenic markers were higher in wild type cells compared to KO cells (Fig. 11C). This suggests that osteogenic differentiation from mouse ES cells is impaired in the absence of calreticulin.

**Chondrogenic potential of calreticulin KO cells**

Thus far, we found that calreticulin deficiency leads to a reduction in osteogenic differentiation. Then, the question arises as to whether these calreticulin KO cells have an increase potential to differentiate towards other lineages that are also derived from mesenchymal origin such as chondrocytes. To address this question, the expression of chondrogenic markers, Aggrecan and collagen 2 (Col 2) were examined in these cells. Col 2 is the principle component of cartilage; while aggrecan is the predominant proteoglycan of cartilage (Fraser et al. 2003). Figure 12 shows that differentiated day 21 nodules from calreticulin KO cells expressed higher levels of Col 2 and Aggrecan compared to their calreticulin wild type counterpart. Moreover, Alcian blue staining revealed that calreticulin KO cells exhibit a stronger blue staining for cartilage extracellular matrix than R1 cells. Thus, this data suggest that the absence of calreticulin leads to higher chondrogenic potential in ES cells.

**Nuclear localization of Runx2 and OSX is impaired in the absence of calreticulin.**

Since Runx2 and OSX are critical transcription factors required for the regulation of osteogenic differentiation and early ES cell commitment to the osteoblastic lineage, their nuclear localization is crucial for their functions. Thus,
Figure 11. Detection of osteogenic differentiation in calreticulin-containing and deficient cells. Calreticulin-expressing mouse ES cells (R1) and calreticulin KO cells were allowed to differentiate for 3 weeks in the presence of RA, B-Gly, AA, and Dex. A. The resulting nodules at day 21 were then fixed in formaldehyde and mineralized (dark) nodules were detected by von Kossa staining. B. Arsenazo III results showed a 7-fold decrease in the extracellular Ca2+ content in the KO cells compared to WT cells (* represents p < 0.05). C. The relative mRNA expression of osteogenic markers (Runx2, OSX, BSP) was detected by Real-time PCR throughout the process of differentiation. All of the three markers were found to have lower expression levels in KO cells compared to wild type cells (** p < 0.01 for all R1 values vs KO values). Conditions were performed in triplicates, data are presented as means ± SD of three independent experiments.
**Figure 12. Increased chondrogenic potential in calreticulin-deficient cells.**  
A. Differentiated day 21 wild type and KO cells were stained with Alcian blue. KO cells had more intense blue staining compared to that in WT cells. B. Real-time PCR analysis revealed that KO cells also expressed higher levels of chondrogenic markers (Col 2 and aggrecan) than WT cells. Conditions were performed in triplicates, data are presented as means ± SD (* represents p < 0.05).
**Figure 12**

**A**

R1  
KO

**B**

**Col 2**

<table>
<thead>
<tr>
<th>Relative mRNA Expression</th>
<th>R1</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

* p < 0.05

**Aggrecan**

<table>
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<th>R1</th>
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<td>30</td>
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*
we performed immunofluorescence staining for Runx2 and OSX in the differentiating R1 and calreticulin-null cells at days 7, 14, and 21. Interestingly, most of Runx2 and OSX are found to be cytosolic in calreticulin KO cells, whereas they were nuclear in wild type cells (Fig. 13). This suggests a prominent role of calreticulin in the nuclear localization of these transcription factors in normal osteogenic differentiation.

**Calreticulin affects osteogenic differentiation via its Ca\(^{2+}\) buffering activity**

Calreticulin is important for its role in intracellular Ca\(^{2+}\) homeostasis through its Ca\(^{2+}\) buffering function. Studies have shown that the absence of calreticulin leads to a reduced Ca\(^{2+}\) store in the ER and thus a smaller releasable pool of Ca\(^{2+}\) from the ER (Arnaudeau *et al.* 2002; Mery *et al.* 1996). To examine whether calreticulin exerts its effect in osteogenesis through this function, the intracellular Ca\(^{2+}\) level was manipulated using either Ionomycin (a Ca\(^{2+}\) ionophore) to increase the cytosolic Ca\(^{2+}\) level in the calreticulin-deficient cells; or BAPTA-AM (Ca\(^{2+}\) chelator) to decrease it in the wild type cells. In the case of the KO cells, the increase in the cytosolic Ca\(^{2+}\) enhanced the level of extracellular mineralization and increased the mRNA expression of osteogenic markers (Runx2, OSX and BSP) (Figs. 14A and B). Furthermore, the increase in Ca\(^{2+}\) level restored the nuclear localization of Runx2 and OSX in calreticulin KO cells (Fig. 14C). In the case of the wild type cells treated with the Ca\(^{2+}\) chelator BAPTA-AM, the level of extracellular mineralization and the expression of osteogenic markers were lowered compared to the non-treated controls (Figs. 14A and B). Lower cytosolic Ca\(^{2+}\) disrupted the nuclear localization of Runx2 and
Figure 13. Immunofluorescence staining of the osteogenic markers, Runx2 and OSX in calreticulin wild type and KO cells. At days 7, 14 and 21 of differentiation, nodules were fixed in 3.7% formaldehyde, permeabilized, and labelled for the transcription factors Runx2 (A) or OSX (B). PI was used to identify the nuclei. Both Runx2 and OSX were found to be mainly localized in the cytosol in the KO cells, while in the wild type cells Runx2 and OSX were nuclear. Scale bar division represents 10 µm.
Figure 13

A

Runx2  PI  Runx2  PI  Runx2  PI

R1

KO

Day 7  Day 14  Day 21

B

OSX  PI  OSX  PI  OSX  PI

R1

KO

Day 7  Day 14  Day 21
Figure 14. The effect of changes in cytosolic Ca\textsuperscript{2+} levels on osteogenic differentiation in calreticulin-containing and deficient cells. BAPTA-AM (Ca\textsuperscript{2+} chelator) or ionomycin (Ca\textsuperscript{2+} ionophore) was used to either lower or increase the cytosolic Ca\textsuperscript{2+} concentration. **A.** von Kossa staining for mineralized nodules showed an increase in mineralization level in KO cells treated with ionomycin; while decreasing the cytosolic Ca\textsuperscript{2+} concentration in R1 cells by treatment with BAPTA-AM decreased the level of mineralization compared with their non-treated controls. **B.** Arsenazo III staining revealed that BAPTA-AM treatment reduced the extracellular Ca\textsuperscript{2+} contents by more than 2-fold. Calreticulin KO cells treated with ionomycin had more than 4-fold increase in their extracellular Ca\textsuperscript{2+} contents compared to their non-treated controls. **C.** The relative mRNA levels of osteogenic markers were increased with increase in cytosolic Ca\textsuperscript{2+} in KO ionomycin-treated cells, while their expression was decreased in the R1 BAPTA-treated cells. **D.** Immunofluorescence staining for Runx2 and OSX localization after cytosolic Ca\textsuperscript{2+} concentration manipulation showed restoration of the nuclear localization of both transcription factors in KO ionomycin-treated cells, while in R1 BAPTA-treated cells, their nuclear translocation was largely impaired. Scale bar division represents 10 µm. Conditions were performed in triplicates, data are presented as means ± SD of three independent experiments (* represents p < 0.05, ** p< 0.01).
OSX; in BAPTA-AM-treated wild type cells, the localization of these osteogenic transcription factors became cytosolic (Fig. 14C). These data suggest that calreticulin exerts its effect on osteogenic differentiation from mouse ES cells via its Ca\textsuperscript{2+} buffering function.

**The involvement of calreticulin’s functional modules in osteogenesis**

Calreticulin has three structural domains (N, P and C) that comprise two functional modules, one responsible for its chaperoning activity ([N+P]) and the other ([P+C]) that carries out the Ca\textsuperscript{2+} buffering function of the protein (Michalak et al. 1999; Michalak et al. 2009; Nakamura et al. 2001). In order to elucidate which structural domain may be involved in calreticulin’s modulation of osteogenesis, we employed two cell lines: calreticulin KO ES cells expressing either its chaperoning [N+P] functional module or its Ca\textsuperscript{2+} buffering [P+C] functional module). These two cell lines were previously utilized in our laboratory to study the involvement of calreticulin’s functional modules in adipogenesis (Szabo et al. 2008). However, experiments using these two cell lines in the present study did not yield consistent results. It appears that both cell lines are needed to be re-established/recloned; hence, this work has been deemed beyond the scope of this thesis.

**DISCUSSION**

**Different cell lines have different osteogenic potential**

The hanging drop protocol (outlined in Figs. 3 and 4) was previously used in our laboratory for ES cell differentiation into the cardiomyogenic and
adipogenic lineage (Papp et al. 2009; Szabo et al. 2009; Szabo et al. 2008). The first cell line we utilized in our osteogenic protocol was mouse CGR8 cells, one of the most common stem cell lines utilized when feederless conditions are desired. Nevertheless, with only the addition of pro-osteogenic molecules, B-Gly and AA, the hanging drop differentiation protocol had low efficiency in inducing the desired osteogenic cell population from mouse ES CGR8 cells. Although differentiated cells did express osteogenic markers such as Runx2, OSX, OCN, and BSP, functional assay by using von Kossa staining revealed that the nodules had little to no mineral deposition, which is a characteristic of functional osteoblasts. The first step in achieving higher efficacy of the differentiation protocol was to utilize another mouse ES cell line, R1, that is also one of the most extensively used stem cell lines isolated by Andras Nagy (Nagy et al. 1993). Our rationale in choosing another cell line is because different cell lines may have different propensity to differentiate to different mature cell types (Cohen and Melton, 2011). A study by Osafune et al. compared the differentiation potential of 17 human ES cell lines and found that the propensity to differentiate into different cell lines are so markedly different that there was often greater than 100-fold difference in the lineage-specific gene expression in the final differentiated cells (Osafune et al. 2008). This is because more evidence is pointing to the inherent differences between different cell lines (Allegrucci and Young, 2007), for instance, there have been reports on the different genetic and epigenetic stability and growth rate observed in long-term culture (Cowan et al. 2004; Hoffman and Carpenter, 2005). These variations could be due to a variety
of reasons, including culture-related effects, inherent genetic variation, differences in genetic and epigenetic stability, and methodological variations (Allegrucci and Young, 2007). Indeed, in our study, differentiated R1 nodules had higher levels of mineral deposition and expressed higher levels of osteogenic markers compared to those obtained with CGR8 cell line. In addition, experiments using R1 cells also achieved better reproducibility and more consistent results. The difference between CGR8 and R1 could be due to any of the inherent differences mentioned above, thus resulting in differing potentials for osteogenic differentiation.

The effect of cell density on ES cell osteogenic differentiation

To further enrich osteogenic cell populations in the differentiated nodules, I proceeded to manipulate additional aspects of culture conditions. Previous studies have suggested that cell density affects stem cell commitment and differentiation to different lineages (Choi et al. 2010; Hwang et al. 2009; Lorincz, 2006; McBeath et al. 2004; Park et al. 2007). Our study found that lower cell density and smaller EB size enhanced osteogenic differentiation, as evident in the higher expression of osteogenic markers and higher mineral deposition in these nodules. This finding is in accordance with a 2004 study conducted by McBeath et al., in which they discovered that lower cell density allows more cell adhesion and spreading, which leads to a more flattened cell shape and higher RhoA activity, induces osteogenic differentiation (McBeath et al. 2004). One unexpected observation in the present study is that EBs consisting of 1000 cells lead to higher osteogenic differentiation than those of 500 cells (Fig. 5).
According to the findings of McBeath et al. (2004), cell density should inversely correlate with the propensity to differentiate into osteogenic cells. One possible explanation for this phenomenon is that the higher levels of hypoxia in the 1000 cell-EBs could be inducing ES cell to differentiate into the osteogenic lineage. It has been reported that hypoxia, by activating the hypoxia-inducible factor 1-alpha (HIF-1α) pathway, has stimulatory effect on osteoblast development (Wang et al. 2007). However, this is only a speculation since there have also been reports of opposite, inhibitory effects of hypoxia on osteogenesis (D'Ippolito et al. 2006; Park et al. 2002; Salim et al. 2004). Thus, although it is known that oxygen tension is a potent regulator of skeletal mass, whether hypoxia is anabolic or catabolic to the skeletal system remains inconclusive.

The pro-osteogenic effects of B-Gly and AA

While protocols for the differentiation of cardiomyocytes, neurons, or adipocytes from ES cells have been well established for many years (Okabe et al. 1996; Wobus et al. 1991), only recently has their differentiation into osteogenic lineage cells been reported (Phillips et al. 2001; Zur Nieden et al. 2003). Osteoblasts differentiate from a common mesenchymal progenitor that can also give rise to adipocytes, chondrocytes and cardiomyocytes. Evidence exists for the presence of cells belonging to non-osteogenic lineages in a variety of cultures (Zur Nieden et al. 2003). Therefore several studies, including the present one, attempted to enhance osteogenesis in vitro by the use of different combinations of various factors in order to increase the efficacy of ES cell
commitment to the osteoblastic lineage (Table 1). Among these pro-osteoblastic factors, B-Gly figures prominently as it is required as a source of organic phosphate for proper matrix mineralization. B-Gly induces differentiation and decreases proliferation in the osteoblastic cell population (Coelho and Fernandes, 2000). In spite of several studies, which questioned whether or not mineralization induced by B-Gly is an exclusive characteristic of osteoblasts\(^1\), B-Gly is routinely added to the differentiation media at the concentration of 10 mM with AA (Bellows \textit{et al.} 1991). Prior to differentiation, osteoblasts secrete a collagenous matrix. AA was found to be essential for osteoblast differentiation \textit{in vitro} and collagen synthesis (Coelho and Fernandes, 2000). Hydroxyapatite-containing mineral is then deposited on this collagenous matrix if a source of organic phosphate such as B-Gly is provided. Furthermore, the addition of AA and B-Gly upregulated several osteogenic markers such as ALP and OCN mRNA expression (Franceschi and Iyer, 1992). The transcription of OCN was triggered by the addition of AA, which was found to have a major role in the stabilization of Runx2 binding to the OCN gene promoter (Xiao \textit{et al.} 1997). Due to their critical roles in osteogenic differentiation from ES cells, B-Gly and AA were both included in all the tested osteogenic protocols.

\(^1\) Chung \textit{et al.} (1992) have shown that the addition of B-Gly induces mineralization that is nonapatitic (Chung \textit{et al.} 1992). Other studies have also questioned whether exogenous addition of B-Gly induces physiological mineralization (Beresford \textit{et al.} 1993; Khouja \textit{et al.} 1990). However, as illustrated in Results, we have confirmed using TEM that mineralization in our culture is apatitic, which is characteristic of those found in bone.
The role of Dex in skeletogenesis

We examined the effect of several factors reported to enhance osteogenic differentiation: RA, Dex, and PPAR\(\gamma\) inhibitor GW9662. We studied their effects alone, as well as in combination. We also looked at the effect of differential timing of addition of Dex. Dex is a glucocorticoid known for its anti-inflammatory properties and thus it is often included in the treatment regimens of inflammatory diseases (Lian et al. 1997). Although some studies have reported its stimulatory effects in osteogenic differentiation (Leboy et al. 1991; Ogston et al. 2002), others have also pointed to its negative effects in osteogenesis (Smith and Frenkel, 2005; Pereira et al. 2002; Shi et al. 2000). Critical factors that may influence such effects of Dex is the maturity and density of the differentiating cells in culture (Mikami et al. 2011). It has been shown that Dex can enhance adipogenesis from mesenchymal stem cells by inducing the expression of the critical adipogenic transcriptional factor, PPAR\(\gamma\) and repressing the expression of the osteogenic transcription factor, Runx2 (Pereira et al. 2002; Shi et al. 2000). On the other hand, Dex can have stimulatory effects on osteogenic differentiation by increasing the expression as well as activity of the enzyme ALP (Ogston et al. 2002). It is also interesting to note that Dex is required to be added in the media continuously to induce maximal osteogenesis; removal of Dex from the culture may cause osteogenic lineage cells to dedifferentiate (Porter et al. 2003). In our study, Dex did indeed enhance osteogenic differentiation as demonstrated by higher levels of mineralization and expression of osteogenic markers. However, in our hands, late addition led to better results than early addition of Dex. This is
because early addition of Dex was found to promote more ES cells to commit to the adipogenic lineage instead of osteogenic lineage, which is achieved through differential temporal regulation of OSX by Dex. This has been demonstrated by Mikami et al. (2011). In their study, early Dex treatment was found to promote adipogenesis, while late addition of Dex promoted osteogenesis in the same cultures (Mikami et al. 2011).

**RA and osteogenesis**

During ES cell differentiation, RA has also been implicated as an important factor to be added to the cultures shortly after EB formation but before further differentiation; it has been shown to induce cells to undergo adipogenesis and neurectoderm differentiation (Duester, 2008). RA signalling is vital for organogenesis in utero and the maintenance of adult organs. (Duester, 2008; Niederreither and Dolle, 2008). RA is a steroid hormone and it exerts its effects on cell differentiation and metabolism by binding to its nuclear receptors, the retinoic X and the RA receptors (Chawla et al. 2001; Mark et al. 2006). It has been recently demonstrated that RA signalling plays an important role in regulating myogenic and hepatic progenitor cell proliferation (Huang et al. 2009; Zhu et al. 2009). It remains controversial regarding the effects of RA on chondrogenesis and osteogenesis (Wan et al. 2006; Wang et al. 2008; Weston et al. 2000). RA has been shown to positively regulate chondrogenesis by increasing the activity of bone morphogenetic protein 2 (BMP2) and inducing the expression of collagen X (Col X) (Zhang et al. 2010). It has also been suggested
that RA promotes osteogenesis by stimulating the expression of Runx2 (Dingwall et al. 2011). This is accomplished by RA interacting with Smad1 and thus enhancing BMP2 signalling (Li et al. 2003). In addition, RA is important in the development of hypertrophic chondrocytes (Drissi et al. 2003). In our study, the addition of RA during the floating stage of differentiation did in fact enhance mineralization as well as the expression of Runx2 and OCN. RA is important for early osteogenic lineage commitment by upregulating Runx2 expression and inhibiting PPARγ (Shao and Lazar, 1997) and Dex enhances later osteoblast differentiation and maturation through its regulation of ALP and OSX activity and expression. Thus, the combined treatment of RA and Dex leads to higher enrichment of osteogenic lineage cells than either addition alone.

**PPARγ: a positive regulator of adipogenesis and negative regulator of osteogenesis**

Several studies have indicated the inhibitory role of PPARγ in osteogenic differentiation (Jeon et al. 2003; Kawai and Rosen, 2010; Lecka-Czernik et al. 2002; Takada et al. 2007), reviewed in (Lecka-Czernik and Suva, 2006). PPARγ is a critical transcription factor that positively regulates adipocyte differentiation and negatively regulates osteoblast formation. *In vivo*, the decreased number of osteoblasts and rate of bone formation that accompanies aging inversely correlates with an increase in number of adipocytes and fat in the bone marrow (Lecka-Czernik and Suva, 2006). This inverse relationship between osteoblast and adipocyte differentiations and their common mesenchymal origin makes PPARγ an obvious candidate to inhibit in order to enhance osteogenic
differentiation from human mesenchymal stem cells (Krause et al. 2010). Inhibitions of this transcription factor have been done using either the potent inhibitor GW9662 or siRNA-mediated inhibition in ES cells (Yamashita et al. 2006). In our study, we also observed an enhancement in osteogenic differentiation from ES cells with the addition of 1 µM of GW9662; although this was not as prominent as the osteogenic enhancement achieved with the addition of RA and (late) Dex. Furthermore, PPARγ is a transcription factor that affects the transcription of several other genes in addition to adipogenic markers. Due to this pleiotropic effect, its inhibition may have other nonspecific downstream effects.

Of all of the 6 tested osteogenic protocols (Fig. 4), consisting of osteogenic factors alone and in combination, with varying timing of addition, RA and late addition of Dex yielded the most pronounced enhancement of osteogenic differentiation based on the results of mineralization assays and the expression of osteogenic markers. Thus, our findings provide valuable guidelines into fine-tuning of culture condition to differentiate mouse R1 ES cells to osteoblastic cells in vitro.

**Roles of calreticulin throughout differentiation**

The second goal of the present work was to determine whether the ER luminal protein, calreticulin, has a role in osteogenic differentiation from mouse ES cells in vitro. There is limited data on the expression and function of calreticulin in osteogenic differentiation. Previous work from our laboratory using transgenic mice containing a Green Fluorescent Protein (GFP) reporter gene
under the control of the calreticulin promoter found that the calreticulin gene is activated in the developing bone (unpublished data, Opas lab). These data suggest an unknown and possibly important role of calreticulin in skeletal development. In the current study, the expression profile of calreticulin in the current cell model gives insight into its functions in osteogenic differentiation. The upregulation of calreticulin from day 0 to day 14 suggests that it has a role in early steps of differentiation such as ES cell commitment to the osteogenic lineage. Indeed, it was found that the Ca\(^{2+}\) buffering function of calreticulin is needed for the proper nuclear translocation for critical osteogenic transcription factors Runx2 and OSX. The nuclear translocation is observed as early as day 7 in our system and it is well known in the literature (Duplomb et al. 2007a; Long, 2012) that the activation of these two transcription factors are required early in differentiation. The abundance of calreticulin expression near day 21 suggests its further role in later differentiation such as mineralization. As demonstrated by Somogyi et al. (2002), calreticulin was found to be present in the extracellular matrix of developing odontoblasts in rats (Somogyi et al. 2003). It is thought that calreticulin’s Ca\(^{2+}\) buffering function may be important for the process of mineralization. Others have also found that calreticulin is upregulated during the mineral deposition phase for ameloblasts, during which these cells require a large amount of Ca\(^{2+}\) (Hubbard, 1996).

**Osteogenic marker expression**

The expression patterns of osteogenic markers Runx2, OSX, and BSP in both calreticulin wild type and KO cells are characteristic of those observed in
vitro (Chou et al. 2005; Duplomb et al. 2007a), albeit calreticulin KO cells displayed significantly lower osteogenic potential. The expression patterns of these osteogenic markers can be explained by their functions in osteogenesis. The osteo-chondroprogenitor cell becomes committed to the osteoblastic cell lineage under the influence of Runx2 and OSX (Karsenty, 2008; Komori, 2008; Long, 2012). Runx2 is a master regulatory gene required to control the expression or activity of several key osteogenic transcription factor (OSX among others) or proteins such as BSP, throughout the process of ES cell osteogenic differentiation (Ducy et al. 1997). OSX is also required during differentiation to activate a repertoire of genes such as Col 1, OCN, osteonectin, osteopontin and BSP (Long, 2012). Since Runx2 and OSX are transcription factors that are needed for the ES cell commitment to the osteogenic lineage as well as activation of the transcription of important extracellular matrix proteins; thus, they are upregulated earlier in the differentiation process. While BSP is a component of the mineralized tissue, it is expressed later in the differentiation sequence.

**Increased chondrogenic potential in calreticulin deficient cells**

Since the absence of calreticulin leads to decreased osteogenic potential in ES cells, what cell lineage(s) are these cells differentiating into alternatively? Chondrogenic lineage cells are obvious candidates to examine since they are derived from common progenitor cells with osteoblasts. Alcian blue staining and PCR analysis revealed that the absence of calreticulin leads to the higher
populations of chondrocytes. It is widely known that Runx2 has an important role for chondrocyte maturation (Komori, 2003), a process that is essential not only in skeletal development but also in bone repair. In the absence of proper chondrocyte maturation, endochondral ossification cannot properly proceed (Mackie et al. 2011). This is because matured chondrocytes secrete factors that are needed for vascular invasion and osteoblast development. In the absence of calreticulin, the nuclear translocation of Runx2 is impaired, thus, it cannot properly carry out its functions in chondrocyte maturation. This could explain why osteogenic differentiation is arrested, as demonstrated by the lower expression of osteogenic markers, and the higher presence of chondrocytes in calreticulin KO cells.

**Impaired nuclear translocation of Runx2 and OSX in KO cells: implications**

Normal osteogenic differentiation of mouse ES cells involves a number of signalling molecules: Runx2, Wnt, insulin/phosphatidyinositol 3-kinase/Akt, bone morphogenetic proteins/Smads, OSX, and many others (Karsenty, 2008). Among them, Runx2 and OSX are indispensable for osteoblastic differentiation; mice with Runx2 or OSX deficiency have a complete lack of bone formation (Komori et al. 1997). Runx2 is a major transcription factor needed for the various stages of osteogenesis such as lineage commitment, proliferation and differentiation (Stewart et al. 1997; Vaillant et al. 2002). Osx is also required for osteoblast
differentiation and bone formation during embryonic development and in postnatal bone growth and homeostasis (Zhou et al. 2010). Thus, the nuclear localization of these two transcription factors is indispensable for their function; and conversely, their cytosolic retention, in the absence of calreticulin, may have prevented their transcriptional activity.

Other studies have also observed cytosolic localizations of these two transcription factors and given insight into how this may impact osteoblast differentiation and maturation. STAT1, a member of the Signal Transducers and Activators of Transcription family, was found to be a negative regulator of osteoblastogenesis. Differentiation of osteoblasts and subsequent bone formation can be prevented by cytosolic retention of Runx2 by STAT1 (Long, 2012). Huang et al. have described that frameshift mutations, which caused at least partially impaired nuclear translocation of Runx2, lead to the phenotype of cleidocranial dysplasia in patients (Huang et al. 2013). Another study by Deepak et al. showed that Tbox3, a previously reported negative regulator of osteoblastogenesis, interferes with Runx2 transcriptional activity. In addition, overexpression of Tbox3 leads to the mis-localization of Runx2 in the cytosol (Deepak et al. 2011). Although it is less known about what may affect the subcellular localization of OSX, Tai et al. showed that OSX nuclear localization and activation is required for early osteoblast development (mesenchymal stem cell and pre-osteoblast stages) and remains nuclear in differentiated osteoblasts; while in non-osteogenic cells such as fibroblasts, OSX remains inactive in the cytosol (Tai et al. 2005).
Calreticulin and the regulation of transcription factors

The finding that calreticulin is required for the proper subcellular location of critical osteogenic factors, Runx2 and OSX is not surprising. This is because calreticulin has previously been shown to be involved in the regulation of subcellular localization of critical transcription factors in other cell lineages. For instance, our laboratory has shown that calreticulin impairs the nuclear translocation of PPARγ, a critical transcription factor in adipogenesis (Szabo et al. 2008). In vitro, calreticulin was found to bind to several transcription factors that contain the KxFFKR amino acid sequence, including PPARγ and the vitamin D receptor (Gurland and Gundersen, 1995; Wheeler et al. 1995). In cardiomyogenesis, calreticulin is needed for the proper nuclear translocation of MEF2C and NFAT. Calreticulin affects calcineurin activity through the control of ER Ca^{2+} release (Lynch and Michalak, 2003). Thus, the nuclear import of NFAT is impaired in calreticulin null cells and this can be restored by re-expression of calreticulin (Mesaeli et al. 1999). Another critical transcription factor for cardiomyogenesis, MEF2C (Lin et al. 1997), does not translocate to the nucleus in the absence of calreticulin either. This is also through the effect of ER Ca^{2+} release and calcineurin activity (Li et al. 2002; Lynch et al. 2005).

FUTURE DIRECTIONS
The involvement of calcineurin-NFAT pathway downstream of calreticulin in its regulation of osteogenesis
Thus far, we discovered a novel role of calreticulin in the osteogenic differentiation from mouse ES cells via its Ca\textsuperscript{2+} buffering function. The nuclear translocation of Runx2 and OSX in the differentiating nodules was affected by manipulation of intracellular Ca\textsuperscript{2+} concentrations. Calreticulin affects several Ca\textsuperscript{2+} signaling pathways; one in particular, the calcineurin-NFAT pathway, has been shown to be critical in osteogenesis. The expression of constitutively nuclear NFATc1 variant in osteoblasts causes the development of high bone mass, massive osteoblast overgrowth, and enhanced osteoblast proliferation (Winslow \textit{et al.} 2006). Sun \textit{et al.} (2005) discovered that calcineurin deficient mice show diminished bone formation and severe osteoporosis; while calcineurin inhibition reduces osteoblast differentiation (Sun \textit{et al.} 2005). Previous findings also showed that calreticulin is an upstream regulator of calcineurin activity (Lynch and Michalak, 2003). To further investigate whether calcineurin is downstream of calreticulin in its regulatory role in osteogenesis, we utilized the non-osteogenic calreticulin-null cells stably transfected with constitutively active calcineurin (cell line denoted “CN”). When differentiated under osteogenic conditions, preliminary data showed that CN cells displayed osteogenic potential comparable to that of calreticulin-containing WT cells (Fig. 15).

Calcineurin, a serine-threonine phosphatase activated by Ca\textsuperscript{2+}, while not considered to have transcriptional activity \textit{per se} (Hogan \textit{et al.} 2003; Hyatt \textit{et al.} 1990), dephosphorylates NFAT proteins (Crabtree, 1999). Dephosphorylated NFATs, with the exposed nuclear localization sequences, then rapidly enter the nucleus (Beals \textit{et al.} 1997) and activate transcription of their target genes. To
firmly establish the involvement of the calcineurin-NFAT pathway in calreticulin’s effects in osteogenesis, we inhibited the nuclear translocation of NFATc using the chemical A-285222. A-285222 specifically inhibits the Ca\(^{2+}\)-induced dephosphorylation of NFAT by calcineurin and its nuclear translocation without inhibiting calcineurin activity (Djuric et al. 2000; Trevillyan et al. 2001). Inhibition of either calcineurin or NFAT in both R1 and CN cells lines decreased their ability to undergo osteogenesis as measured by a decrease in mineralization and downregulation of osteogenic markers (Fig. 15). Further work would be required to decipher how calreticulin, via affecting the calcineurin-NFAT pathway, regulates osteogenesis. It is very plausible that this signalling cascade is responsible for the decreased expression of osteogenic markers, Runx2, OSX, and BSP in the calreticulin-null cells. Furthermore, an interesting report by Li et al (2011) demonstrated that calcineurin-NFAT signalling affects c-Src expression, which would have a negative effect on cell adhesive properties that are important in osteogenesis.

**STAT1: cytosolic attenuator of Runx2**

Since the absence of calreticulin impairs the nuclear translocation of Runx2, we further investigated how calreticulin, via its Ca\(^{2+}\) buffering activity, affects this phenomenon. STAT1 is a signalling molecule that, when phosphorylated at Y701, dimerizes and translocates to the nucleus. However,
Figure 15. The involvement of calcineurin-NFAT pathway downstream of calreticulin in osteogenesis. A. Non-osteogenic calreticulin-null cells stably transfected with constitutively active calcineurin (cell line denoted “CN”) showed increased mineral deposition, comparable to that in wild type cells. Arsenazo III assay revealed that CN cells had increased extracellular Ca\(^{2+}\) content. B. In addition to increased mineralization, CN cells also had upregulated expression of osteogenic markers (Runx2, OSX, BSP). A and B. Wild type and CN cells treated with either calcineurin inhibitor (cyclosporin, or CsA) or NFAT inhibitor (A-285222) had lowered mineralization and expression of osteogenic markers, further confirming the involvement of calcineurin and NFAT downstream of calreticulin. Conditions were performed in triplicates, data are presented as means ± SD of three independent experiments (* represents p < 0.05, ** p< 0.01).
Figure 15

A

Control  CsA  A-285222

R1

CN

B

RUNX2

OSX

BSPIII

* p < 0.05  ** p < 0.01
when the Y701 residue of STAT1 is not phosphorylated, the protein resides in the cytosol (Darnell, Jr. et al. 1994) where it binds Runx2, and the complex is retained in the cytosol (Kim et al. 2003). Differentiation of osteoblasts and subsequent bone formation can be prevented by cytosolic retention of Runx2 by STAT1 (Kim et al. 2003; Long, 2012; Xiao et al. 2004). Thus, it is possible that calreticulin affects localization of STAT1. Immunostaining revealed that the majority of STAT1 was localized to the nuclei in calreticulin-containing R1 cells, while it was predominantly cytosolic in non-osteogenic calreticulin KO cells (Fig. 16). Moreover, changes in intracellular [Ca^{2+}] altered STAT1 localization: in R1 cells, after depleting cytosolic Ca^{2+} with BAPTA-AM, STAT1 resided in the cytoplasm. In contrast, after ionomycin treatment to increase intracellular [Ca^{2+}] in KO cells, STAT1 nuclear translocation was restored (Fig. 16).

Future work could investigate how the intracellular Ca^{2+}, modulated by calreticulin, has a role in STAT1 localization. It is highly plausible that calcineurin may also have a role in modulation of STAT1 subcellular localization, possibly by affecting its phosphorylation status (Y701) indirectly.

**Calreticulin and OSX nuclear localization**

In contrast to Runx2, less is known about the regulation of subcellular localization of OSX. The present study uncovered a novel regulator, calreticulin, in OSX nuclear translocation. Calreticulin has previously been shown to have a role in nuclear trafficking of various proteins (Coppari et al. 2002; Holaska et al. 2002). A study by Holaska et al. (2002) illustrated the ability of calreticulin to directly bind to the DNA binding domain of the glucocorticoid receptor and
Figure 16. Calreticulin regulates the subcellular localization of STAT1. STAT1, a cytosolic attenuator of Runx2 when unphosphorylated at Y701, was found to be affected by the presence or absence of calreticulin. Immunofluorescence staining on day 21 differentiated nodules revealed that in the presence of calreticulin, majority of STAT1 is localized in the nucleus. Calreticulin deficient cells was found to have the majority of STAT1 in the cytosol instead. This observation was Ca$^{2+}$-dependent: increasing intracellular Ca$^{2+}$ by ionomycin in calreticulin KO cells restored the nuclear localization of STAT1; while decreasing Ca$^{2+}$ concentration by BAPTA-AM led to cytosolic retention of STAT1 in calreticulin containing R1 cells. Scale bar division represents 10 µm.
Figure 16

<table>
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<td>R1</td>
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R1: BAPTA-AM
KO: ionomycin
facilitate its nuclear export. This event was found to be Ca\(^{2+}\) dependent. Furthermore, it was uncovered that the nuclear import of protein disulfide isomerase, ERp57, may be facilitated by calreticulin and STAT3 (Coppari \textit{et al.} 2002). Thus, it is highly plausible that calreticulin affects OSX subcellular localization by directly binding to it and facilitating its nuclear import. This phenomenon could be addressed in future studies by performing immunoprecipitation with OSX to investigate whether calreticulin physically associates with it.
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APPENDIX

Below are the cycle numbers (Ct values) for one representative experiment and the corresponding amplification curve, melting curve for each figure.

**Figure 5**

<table>
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<tr>
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