Transcription Factor FOXC1 Deregulates BMP-SMAD Signalling During Osteoblast Differentiation

by

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Abstract

Skeletal development is a tightly regulated process that continues through adulthood in the form of bone remodeling. Many bones of the appendicular and axial skeleton develop using a cartilage intermediate in a process called endochondral ossification. The cranial and flat bones of the skeleton develop directly from osteoblasts through intramembranous ossification. Among the important signalling pathways regulating bone formation is BMP-SMAD signalling. BMP ligands bind transmembrane receptors that activate receptor SMAD proteins. These activated proteins enter the nucleus and bind various cofactors to increase target specificity and regulate gene expression during bone development.

Forkhead box (FOX) transcription factors all contain a forkhead domain capable of DNA binding in the major and minor groove of the DNA double helix. FOX proteins are involved in a diverse array of biological processes from homeostasis to organ and tissue development and cell proliferation. TGF-β and BMP signalling directly interact with FOX proteins for target specificity and regulation. FOXC proteins are a subfamily of FOX proteins with overlapping functions throughout development. Vascular, renal, and eye development proceed optimally through tightly regulated FOXC activity. FOXC proteins also contribute to proper bone development and patterning by interaction with TGF-β-SMAD and BMP-SMAD signalling. FOXC1 and FOXC2 are both capable of directly interacting with common SMAD4. When FOXC1 is ablated in mice, errors in endochondral and intramembranous ossification develop leading to small, misshapen endochondral bone while many intramembranous bones are completely missing. Despite understanding that effective BMP-induced bone development depends on expression of FOXC1, the nature and mechanism of the relationship is not known.

The present research aims to discover the mechanism through which FOXC1 impacts bone formation. This work explores FOXC1's impact on a SMAD binding site isolated from the SMAD target gene *Id1* called the BMP Responsive Element (BRE) with and without BMP-4 induction. FOXC1 constructs missing functional regions are tested against wildtype FOXC1 function on BRE activation to determine regions crucial to BRE regulation. Finally, mouse myoblast cells stably expressing FOXC1 are used to evaluate what effect FOXC1 has on BMP-induced osteoblast transdifferentiation of myoblast cells.

This work reports FOXC1 inhibits BMP-induced BRE activation through interaction with the BMP-SMAD pathway. The N-terminal activation, inhibitory, and DNA binding domains of FOXC1 are all important for this inhibitory activity. Ectopic FOXC1 production in myoblasts likewise inhibits endogenous *Id1* expression, though not other BMP-SMAD target genes. Osteoblast markers Alpl and Col1a1 are upregulated in cells stably expressing *FOXC1* independent of BMP-4 treatment.

FOXC1 appears to inhibit basal and BMP-4 induced *Id1* expression. FOXC1 otherwise may not directly impact BMP-SMAD target expression, suggesting FOXC1 specifically targets *Id1* expression and not BMP-SMAD signalling globally. Transdifferentiation of myoblast cells ectopically expressing *FOXC1* can proceed without BMP-4 induction, indicated by *Col1a1* and *Alpl* upregulation. These findings suggest FOXC1 may play an important role in the early stages of osteoblastogenesis.

Preface

This thesis contains original work by Jordan Caddy. No part of this work has previously been published.

Acknowledgements

I would like to thank my supervisor, Fred Berry, for his excellence in research that was an example for me to emulate. His patience and encouragement throughout my project motivated me when I felt overwhelmed or confused with the project or the results I was finding. Whenever I approached him with a question regarding my research he knew what questions would set me on the path to answer it myself, and that augmented my ability for self-directed learning exponentially. I would also like to thank the other members of my lab, Rotem Lavy and Leiah Luoma, who were exceptional in their example as scientists and were always more than willing to work with me through a problem I encountered in my experimental results or protocols. They helped make my time in the lab very enjoyable. I would also like to thank a former member of the lab, Freda Mirzayans, who was instrumental in the early success of my project and served as the resident expert in every experiment I ran before her retirement.

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List of Abbreviations

BMP – Bone Morphogenetic Protein

SMAD – SMA/MAD Protein

FOX – Forkhead Box Transcription Factor

DNA – Deoxyribonucleic acid

TGF-β – Transforming Growth Factor beta

BRE – BMP Response Element

Id – Inhibitor of Differentiation

Alpl – Alkaline phosphatase ligand

Col1a1 – Collagen subunit 1 alpha 1

ECM – Extracellular matrix

bHLH – Basic helix loop helix protein

Runx2 – Runt-related transcription factor 2

Ocn – Osteocalcin

Dlx – Distal-like Homeobox protein

Opn – Osteopontin

Osx – Osterix

Bsp – Bone Sialoprotein

Wnt – Wingless type

BMPR – BMP receptor protein

Zranb2 - Zinc finger, RAN-binding domain containing 2

TBX1 – T-box Transcription Factor 1

NF-KB – Nuclear Factor kappa B

Hey1 – Hairy/enhancer of split related with YRPW motif 1

Ihh – Indian Hedgehog

Msx2 – Msh Homeobox 2

C2C12 – mouse myoblast cell line

rhBMP – recombinant human BMP

DBD – DNA-binding domain

FHD – forkhead domain

NLS – nuclear localization signal

AD – activation domain

IHD – inhibitory domain

BLBC – basal-like breast cancer

PITX2 – paired-like Homeobox 2 or pituitary Homeobox 2

EGFR – Epidermal growth factor receptor

ch – congenital hydrocephalus

SOX9 – Sex determining region Y-box 9

TLE4 – Transducin-like enhancer of split 4

IL-8 – Interleukin 8

FGF – Fibroblast growth factor

HH – Hedgehog

FBS – Fetal bovine serum

TE – Trypsin-EDTA buffer

EDTA – ethylenediamine tetra-acetic acid

BRE-luc – BRE-luciferase reporter

DMEM – Dulbecco's modified eagle media

WT – Wildtype

PBS – Phosphate buffer saline

HCl – hydrochloric acid

LARII – Luciferase assay reagent II

DTT – Dithiothreitol

PMSF – Phenylmethylsulfonyl fluoride

PIC – Protease inhibitor cocktail

BSA – Bovine serum albumin

WB – Western blotting

SDS – sodium dodecyl sulphate

TBST – Tris-buffered saline with Tween 2.0

RNA – ribonucleic acid

BCIP/NBT – 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium

qRT-PCR – quantitative reverse transcriptase polymerase chain reaction

EV – Empty vector

XP – XPRESS

BMP2K – BMP-2 inducible kinase

FGFR – fibroblast growth factor receptor

N.S. – Not significant

N.A. – Not applicable

R-SMAD – Receptor SMAD

Co-SMAD – Common SMAD

pSMAD – phosphorylated SMAD

FLNA – Filamin-A

CKD – Chronic Kidney Disease

TNF-α – Tumor necrotic factor alpha

VSMC – vascular smooth muscle cell

CoIP – Co-immunoprecipitation

ChIP – Chromatin immunoprecipitation

ES cells – Embryonic stem cells

miR – microRNA

EMT – Endothelial to mesenchymal transition

Chapter One: Introduction

1.1 Bone Development and Patterning

1.1.1 *Roles of the Skeleton*

The skeleton has long been known to serve many functions in biology: it is a frame for the structure and support of the vertebrate body; it protects vulnerable systems from physical harm; it has joints for movement and is the anchor that muscles pull and push against to affect movement and stability. Additionally, it ensures homeostasis of biologically important minerals such as fluoride, calcium and phosphorus and produces red blood cells for muscle oxygenation (Armstrong & Singer, 1968; Rodan et al., 1968). More recently Osteocalcin (Ocn), a bone matrix protein, was shown to regulate endocrine function; adding the skeleton as an endocrine gland (Martin, 2007). Although bone development, remodeling, and repair are constantly being investigated and disorders and treatments are researched extensively, there is much yet to understand about the skeleton. As the various aspects of the skeleton are further studied, the understanding we gain may inform innovative solutions to problems in the treatment and prevention of skeletal diseases.

The skeleton is developed through two different processes: endochondral and intramembranous ossification (Karsenty et al., 2009; Long & Ornitz, 2013; Percival & Richtsmeier, 2013). Both methods of bone formation require tight gene and signalling pathway regulation for proper timing and growth of developing bone (Eames, de la Fuente, & Helms, 2003; Long & Ornitz, 2013; Percival & Richtsmeier, 2013; Ting et al., 2009; Yeung Tsang et al, 2014).

1.1.2 *Endochondral Ossification*

Bones making up the axial and appendicular skeleton arise from endochondral ossification as do the base and posterior skull (Berendsen & Olsen, 2015; Long, 2012; Long & Ornitz, 2013). The axial and appendicular skeleton is derived from mesenchyme and the endochondral cranial bones originate from neural crest cells. Mesenchymal or neural crest cells likely condense through Ca^{+} independent cell-to-cell adhesion facilitated by N-Cadherin; this condensation results in the formation of the approximate shape of the destined bone (DeLise et al., 2000; Long & Ornitz, 2013; Radice et al., 1997). Chondrogenic mesenchymal condensation also requires Bone Morphogenetic Protein 2 and 4 (BMP-2/4) signalling and regulation by *Hoxa13* and *Hoxd13* (Fromental-Ramain et al., 1996; Kume et al., 1998). Cells within the condensed mass then differentiate to chondrocytes and cells located on the exterior of the mass form the perichondrium (Long & Ornitz, 2013). Chondrocytes within the condensation continually produce a cartilage matrix made up of proteoglycans and three types of collagen (II, IX, and XI) while proliferating, causing the mass to grow linearly and taking the shape of the destined bone further. The innermost chondrocytes eventually stop proliferating and mature into hypertrophic chondrocytes that excrete type X collagen as the cartilage calcifies (Roach, 1992; Yeung Tsang et al., 2014). As chondrocyte maturation proceeds, chondrocytes closer to the termini of the developing bone (growth plates) mature as well (Long & Ornitz, 2013). Hypertrophic chondrocytes are vascularized as cells adjacent to the inside of the perichondrium differentiate to osteoblasts, form the bone collar by secreting type I collagen and create extracellular matrix (ECM) (Roach, 1992). Vascularization of the inner cartilage matrix allows the invasion of chondroclasts; which break down chondrocytes, and preosteoblasts that mature to osteoblasts and secrete ECM to form trabecular bone (Long & Ornitz, 2013; Maes et al., 2010;

Yeung Tsang et al., 2014). Alternatively, chondrocytes within the cartilage matrix are capable of transdifferentiation to preosteoblasts or programmed cell death, assisting progression of endochondral ossification (Jing et al., 2015; Yeung Tsang et al., 2014). As the bone develops, proliferating chondrocytes within the cartilage matrix of the growth plates in turn mature, become vascularized, and transdifferentiate or undergo cell death as preosteoblasts invade the cartilage matrix to create more trabecular bone (Long & Ornitz, 2013; Yeung Tsang et al., 2014). This process continues until the bone reaches maturity.

1.1.3 *Intramembranous Ossification*

In contrast to endochondral ossification, intramembranous ossification does not use a cartilage intermediate during bone development. These bones are flat in appearance and, excepting part of the clavicle, are found in the craniofacial skeleton (Berendsen & Olsen, 2015; Percival & Richtsmeier, 2013). Like endochondral bone, intramembranous bone begins as mesenchymal or neural crest cells condense. However, expression patterns differ between the two types of ossification, and condensations destined for intramembranous formation are preosteogenic rather than prechondrogenic (Percival & Richtsmeier, 2013). Condensed cells rapidly proliferate and eventually differentiate to *Runx2*- and *Col1*-producing preosteoblasts (Abzhanov et al., 2007; Ting et al., 2009). Radial expansion of the developing bone does not proceed, however, until the osteoblast-secreted ECM begins to be mineralized (Lana-Elola et al., 2007; Percival & Richtsmeier, 2013; Yoshida et al., 2008). Expansion of intramembranous bone occurs by migrating osteoblasts at the osteogenic fronts; though neighbouring mesenchymal cells otherwise destined to become sutural cells to adjoining bones may contribute to osteogenic growth by differentiating to osteoblasts when cell density is low in the osteogenic fronts (Lana-Elola et al., 2007; Ting et al., 2009; Yoshida et al., 2008).

1.1.4 *Gene Expression and Signalling in Differentiating Osteoblasts*

As stated earlier, osteoblasts are formed in endochondral and intramembranous ossification. Proper osteoblast differentiation requires a specific schedule of upregulation and inhibition of genes to ensure proper timing during differentiation (Peng et al., 2004). Inhibitor of differentiation (Id) genes are highly expressed in mesenchymal precursor cells and were initially found to inhibit myogenic differentiation in favor of cellular proliferation by binding basic helix-loop-helix (bHLH) proteins, preventing transcriptional activation of genes involved in myogenic differentiation (Benezra et al., 1990; Kreider et al, 1992). Id genes are initially highly expressed in C2C12 (mouse myoblast) cells in response to treatment with bone morphogenetic proteins 2, 4, 6 or 9 (BMP) (Katagiri et al., 1994; Peng et al., 2004). Following this initial activation is inhibition of Id genes. This inhibition occurs as the expression of other BMP-2/4 targets and osteogenes increase, beginning a cascade of gene expression as precursor cells differentiate to osteoblasts (Katagiri et al., 2002; Peng et al., 2003; Peng et al., 2004).

Runt-related transcription factor 2 (*Runx2*) is expressed in early mesenchymal precursors differentiating into osteoblasts and chondrocytes (Abzhanov et al., 2007; Ducy et al., 1997; Otto et al., 1997; Ting et al., 2009). Its expression is essential in all bone formation as well as bone maintenance: *Runx2*$^{-/-}$ mice exhibit no bone formation, and mice with decreased ability for wildtype RUNX2-DNA binding had reduced postnatal bone formation (Ducy et al., 1999; Komori et al., 1997; Otto et al., 1997). RUNX2 is likewise a crucial transcription factor for osteoblast differentiation and directs transcription of *Collagen 1a1* (*Col1a1*), *Col1a2*, and *Ocn* which contribute to bone matrix formation and bone mineralization, respectively (Beck et al., 2001; Ducy et al., 1997).

Distal-like Homeobox 3 (*Dlx3*) is upregulated during osteoblast differentiation and its protein, DLX3 is recruited with DLX5 to the promoter of *Osteopontin* (*Opn*), another osteoblast marker gene important in ECM formation (Hassan et al., 2004). *Dlx5* has been shown to induce *Osterix* (*Osx* in mice or *Sp7* in humans) expression, both of which are upregulated during osteoblast differentiation (Lee et al., 2003b). Interestingly, DLX5 also has been shown to induce *Runx2* expression, only under specific osteoblast differentiation by BMP-2/4 (Lee et al., 2003a). An illustration of osteoblast marker gene expression profiles based on microarray and genome-wide expression analysis can be seen in Figure 1.1.

Col1a1 is expressed most highly in osteoblasts secreting ECM, though expression is driven in different tissues by tissue-specific transcription factors (Bedalov et al., 1995). Expression begins early in preosteoblasts undergoing maturation and decreases as the ECM mineralizes (Beck et al., 2001). Two COL1A1 subunits trimerize with a COL1A2 subunit to form collagen 1, which makes up the majority of osteoblast ECM (Bedalov et al., 1995; Kern et al., 2001; van der Rest & Garrone, 1991). RUNX2 drives transcription of *COL1A1* in osteoblasts, as does DLX3 and DLX5 (Ducy et al., 1999; Hassan et al., 2004; Tadic et al., 2001).

Preosteoblasts begin expressing *Osx* as the ECM is being formed and *Osx* expression is noted in all trabecular bone and secondary ossification centers (Nakashima et al., 2002). *Osx* is essential to proper osteoblast gene expression and bone formation, as *Osx* null mouse osteoblasts and skeletal elements show reduced levels of *Col1a1*, *Bone sialoprotein* (*Bsp*), *Opn*, and *Ocn* (Baek et al., 2013; Nakashima et al., 2002). RUNX2 can bind the *OSX* promoter and is sufficient to induce expression, though DLX5 can also bind its promoter and drive expression independent of RUNX2 (Lee et al., 2003; Nishio et al., 2006).

Mesenchymal cells express heightened levels of *Alpl* and show increased extracellular Alpl during osteoblastic differentiation and maturation (Lorch, 1949; Rawadi et al., 2003; Sun et al., 2015). *Alpl* expression is induced by BMP-2/4 independently of RUNX2 only when Wingless type (Wnt)/Catenin B signalling is intact (Mbalaviele et al., 2005; Rawadi et al., 2003). ALPL is initially produced in early to mid preosteoblasts as the ECM is forming and serves to remove pyrophosphate ions from the ECM and allow mineralization to proceed (Murshed et al., 2005; Peng et al., 2004; Rawadi et al., 2003), and its extracellular presence is commonly used as a marker for osteoblast differentiation and bone formation (Mirzayans et al., 2012; Peng et al., 2004; Rawadi et al., 2003). Normal levels of extracellular Alpl deposition is necessary for proper bone development as mutations to Alpl that reduce its activity results in Hypophosphatasia with varying degrees of severity (Fedde et al., 1999).

1.2 BMP-SMAD Signalling

1.2.1 *BMP-SMAD signalling pathway*

BMPs were first discovered when demineralized bone matrix was inserted into muscle, resulting in ectopic bone formation (Urist et al., 1970; Van de Putte & Urist, 1965). Osteoblast differentiation proceeds through induction by osteogenic BMPs, including BMP-2/4 (Wang et al., 1988; Wang et al., 1990; Wang et al., 1993). BMP-2/4 ligands dimerize and bind a heterotetramer of BMP Receptor I and II (BMPRI and BMRII) (Fig 1.2) (Yamashita et al., 1995). When the complex is bound by BMP-2/4 ligand, BMPRII phosphorylates BMPRI, which then phosphorylates a receptor SMA/MAD protein (R-SMADs), SMAD1, 5, or 9 (previously SMAD-8) (Massague, 1998; Piek et al., 1999; Tsukamoto et al., 2014; Yamashita et al., 1995).

Figure 1.1

Gene expression changes during osteoblast differentiation under BMP-2/4 induction. Mesenchymal precursors begin undergoing differentiation when treated with BMP-2/4. *Id1* expression is initially high, but decreases sharply when differentiation begins (Peng et al., 2004). *Dlx3*, *Dlx5* and *Runx2* expression begins soon after induction with BMP-2/4. *Foxc1* is initially high in precursor cells, but declines less dramatically than *Id1* as differentiation proceeds (Hopkins, Mirzayans, & Berry, 2015). Dlx3, Dlx5 and Runx2 proteins drive the transcription of *Alpl*, *Col1a1*, *Ocn*, *Opn*, and *Osx* expression (Ducy et al., 1999; Hassan et al., 2004; M. H. Lee et al., 2005; Nishio et al., 2006; Tadic et al., 2001). Figure is modelled after Peng et al 2004.

Figure 1.1

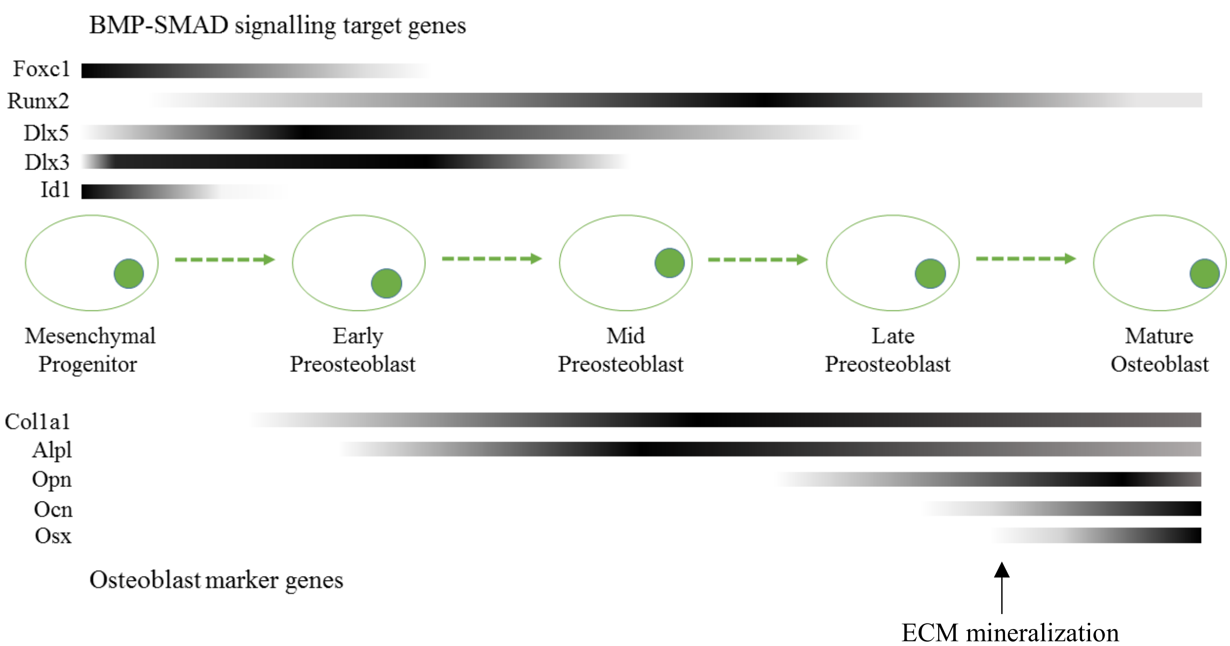
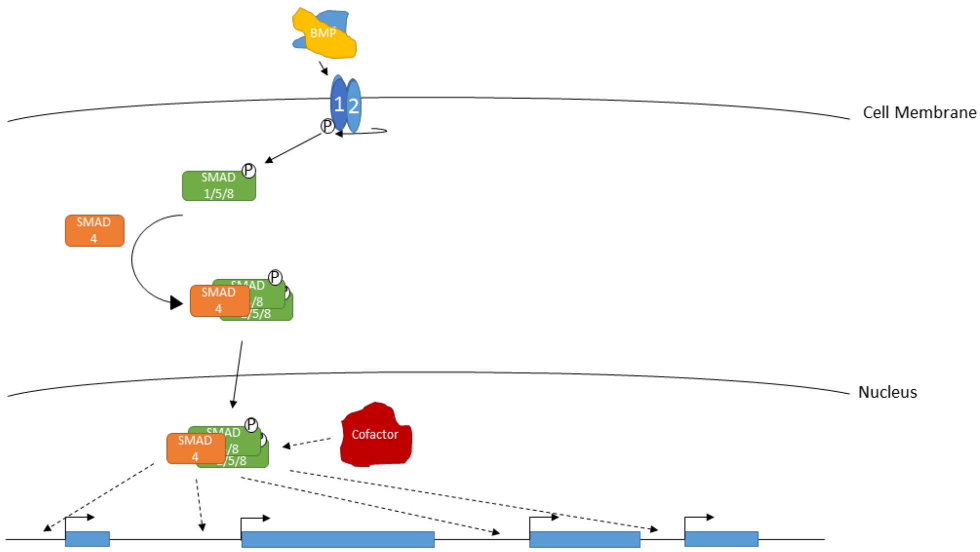

BMP-SMAD signalling target genes
Foxc1
Runx2
Dlx5
Dlx3
Id1
Mesenchymal Progenitor
Early Preosteoblast
Mid Preosteoblast
Late Preosteoblast
Mature Osteoblast
Col1a1
Alpl
Opn
Ocn
Osx
Osteoblast marker genes
ECM mineralization

Figure 1.2

BMP-SMAD signalling in osteoblastogenesis. BMP-2 and -4 can hetero or homodimerize to bind BMPRI and BMPRII heterotetramers. Once bound by a ligand, BMPRII phosphorylates BMPRI, which then phosphorylates and activates an R-SMAD. Two active R-SMADs trimerize with SMAD-4 and enter the nucleus. The SMAD complex then binds cofactors such as DLX5, RUNX2 and FOX proteins to determine specific gene targeting.

Figure 1.2


BMP
1
2
Cell Membrane
SMAD
1/5/8
SMAD
4
SMAD
4
Nucleus
SMAD
4
Cofactor

Phosphorylation to R-SMAD's MH2 domain changes its conformation to a more linear, active state allowing two active R-SMADs (phosphorylated SMADs, or pSMADs) to bind one common SMAD; SMAD-4 (Lagna et al., 1996; Piek et al., 1999). Once bound as a heterotrimer, the SMAD complex enters the nucleus and either binds DNA targets alone or interacts with various cofactors such as RUNX1, RUNX2, DLX5, FOXC1, and FOXC2 to influence target specificity (Fei et al., 2010; Fujita et al., 2006; Hanai et al., 1999; Lagna et al., 1996; Leboy et al., 2001; Liu et al., 1997).

Activated R-SMADs 1 and 5 act redundantly and are crucial to proper bone formation in mice (Retting et al., 2009). Endochondral bone formation is partially affected, but not completely abrogated, when SMAD-4 is knocked out in mice suggesting that phosphorylated R-SMADs are capable of entering the nucleus and affecting target gene expression without binding SMAD-4 (Retting et al., 2009; Zhang et al., 2005). E4F1 also binds with SMAD-4 to inhibit myogenesis (Nojima et al., 2010). Additionally, proteins such as TBX1, NF-κB and ZRANB2 may bind the complex to inhibit DNA binding or transcription (Fulcoli et al., 2009; Hirata-Tsuchiya et al., 2014; Ohte et al., 2012). Finally, SMAD signalling is also regulated by endocytosis inhibition which may change expression of target genes without changing cofactor binding (Heining et al., 2011).

Once in the nucleus, the SMAD complex induces expression of BMP-2/4-inducible genes such as *Id1*, *Runx2*, *Hey1* (*Hairy/enhancer of split related with YRPW motif 1*), *Dlx3*, *Col1a1*, *Indian Hedgehog* (*Ihh*), and *Msx2* (*Msh Homeobox 2*) (Dahlqvist et al., 2003; Hassan et al., 2004; Katagiri et al., 2002; Kern et al., 2001; Leboy et al., 2001; Lee et al., 2000; Lee et al., 2003a; Lee et al., 2003b; Lee et al., 2005; Lopez-Rovira et al., 2002; Mirzayans et al., 2012; Park & Morasso, 2002; Peng et al., 2003; Seki & Hata, 2004; Sun et al., 2013). The protein products of these genes play important roles in BMP-SMAD signalling as well as osteoblast differentiation and maturation. ID1 promotes rapid proliferation while inhibiting myogenic differentiation (Katagiri et al.,1994; Katagiri et al., 2002; Kreider et al., 1992). RUNX2 binds SMAD complex to upregulate downstream BMP-SMAD and osteogenic targets (Kern et al., 2001; Leboy et al., 2001; Nishio et al., 2006). MSX2 balances proliferation and differentiation in preosteoblasts for proper bone patterning (Liu et al., 1999). HEY1 has been shown to induce early osteoblast differentiation and inhibit myogenic differentiation in C2C12 cells (Dahlqvist et al., 2003; Sharff et al., 2009). COL1A1, OSX, OPN, and OCN when induced by BMP-SMAD signalling exit the cell where they produce or mineralize the ECM (Hauschka & Reid, 1978; Kern et al., 2001; Mark et al., 1988).

1.2.2 *BMP Signalling Interacts with other Signalling Pathways*

BMP signalling interacts with many other signalling pathways during development to ensure proper bone development and patterning (Lin & Hankenson, 2011). FGF signalling cooperates with BMP signalling in developing eyelids to ensure proper closure but is antagonistic to BMP signalling in endochondral ossification in the growth plate (Huang et al., 2009; Naski et al., 1998; Yoon et al., 2006). This context-dependent change in relationship also occurs between Wnt and BMP signalling in bone development. Inhibiting Wnt signalling increases BMP-induced ALPL production in C2C12 mouse myoblasts (Fujita & Janz, 2007). However, β-Catenin: a component of canonical Wnt signalling is an essential component in BMP-induced bone formation (Chen et al., 2007).

BMP interacts with Notch and IHH signalling as well. BMP 2/4 treatment of myoblasts induces Notch signalling to inhibit myogenic differentiation through *Hey1* expression (Dahlqvist et al., 2003). Recently, Notch has also been shown to inhibit the cell cycle through p53 and aid BMP-induced chondrocyte hypertrophy (Shang et al., 2016). IHH signalling is also crucial to proper endochondral bone development and is induced by BMP-SMAD signalling to regulate ossification (Minina et al., 2001; Seki & Hata, 2004; St-Jacques et al., 1999).

1.2.3 *BMPs in Clinical Treatment*

Originally, BMPs were isolated from bone and introduced with a collagen delivery system to best promote bone growth in animal studies (Gao et al., 1996). Cloning and expression of recombinant BMPs made it possible for affordable BMP use in clinical treatments (Wang et al., 1990; Wozney et al., 1988). Recombinant BMP-2 and BMP-7 have been used clinically to assist post-surgery healing and regrowth and to promote joining of non-unions in fractures since 2001 with success (Carreira et al., 2014). However, treatment with BMP-2 *in vivo* requires much higher volumes than what exists endogenously for bone development. Also, complications and side effects including: swelling and disorganized bone formation in non-union repair, dysphasia in spinal fusions, and gingival swelling during cleft repair have been reported (Cahill et al., 2009; Neovius et al., 2013; Ritting et al., 2012).

1.2.4 *Gaps in Knowledge*

Despite the research and investigation aimed at understanding the BMP-SMAD signalling pathway, its interaction with other pathways, and how it may be used clinically to improve bone formation and healing, there is much yet to be understood. For example; the clinical results of BMP-2 treatments are varied but reasons for differences in treatment results and complications are yet to be fully discovered. One possible reason for complications in treatment is the high doses of recombinant human BMP-2 (rhBMP-2) required for physiological response (Gibbs et al., 2016). The use of nanogels to localize BMP and protect it from degradation shows promise, though more research is required for its establishment in the clinic.

In addition, new discoveries regarding BMP-SMAD signalling, its contributors and antagonists are continually being pursued and reported in the literature (Hopkins et al., 2015; Rahman et al., 2015; Shang et al., 2016). Despite these advances in our understanding, much is left to uncover regarding BMP signalling and how we may include treatments to use BMP signalling in therapeutic ways.

1.3 Forkhead Box Transcription Factors

A transcription factor containing the fork head DNA-Binding Domain (FHD) was first cloned from *Drosophila melanogaster* in 1989 (Lai et al., 1990; Weigel et al., 1989). As forkhead domain-containing transcription factors were increasingly discovered, a common nomenclature was developed, giving all transcription factors containing an FHD the name Forkhead Box transcription factors (FOX) (Kaestner et al., 2000). There are 19 subfamilies in the FOX family, with each transcription factor assigned to a subfamily based on phylogeny, and numbered therein (Benayoun et al., 2011; Kaestner et al., 2000). Gene expression and protein function in biology and development vary across the subfamilies (Benayoun et al., 2011).

FOX proteins have been shown to interact with several signalling pathways including but not limited to Transforming Growth Factor Beta (TGF-β), and Wingless Type (WNT) signalling (Ahmad et al., 2016; Carlsson & Mahlapuu, 2002; Danesin & Houart, 2012; Mears et al., 1998; Reid et al., 2016). In addition, FOX proteins play crucial roles in development; as misexpression, mutations and deletions have resulted in developmental impairment and disease (Aldinger et al., 2009; French et al., 2014; Hamdan et al., 2010; Horn et al., 2010; Lehmann et al., 2003; Maier et al., 2013; Mirzayans et al., 2000). Finally, some FOX proteins have also been linked to cancer progression/prognosis as well as aging; likely due to their proposed roles in cell proliferation and quiescence (Lam et al., 2013; Omatsu et al., 2014; Ray et al., 2010; Saba et al., 2016; Wang et al., 2012; Wang et al., 2016a).

1.4 FOXC1

1.4.1 *FOXC1 Structure*

Forkhead box Transcription Factor C1 (FOXC1) is a helix-wing transcription factor capable of binding DNA and proteins to affect transcription and regulate various aspects of development (Li et al., 2015; Murphy et al., 2004; Saleem et al., 2001). FOXC1 is capable of entering the nucleus by and binding DNA through its FHD which contains a Nuclear Localization Signal (NLS) at either end of the FHD (Fig 1.3a) (Berry et al., 2002; Saleem et al., 2004). The FHD has a winged-helix-turn-helix motif and is capable of binding DNA in the major groove (bound by α-helix 3) and the minor groove (bound by the side chain of R169 in wing 2) of the DNA double helix at its consensus target: 5’-GTAAATAAA-3’ located within α-helix 3 (Fig 1.3b) (Clark et al., 1993; Murphy et al., 2004; Saleem et al., 2001; Saleem et al., 2004; van Dongen et al., 2000). When bound by FOXC1, DNA bends 112˚, which may result in increased binding affinity for other transcription factors and transcriptional machinery (Saleem et al., 2003). In addition to the FHD, FOXC1 contains two transcriptional activation domains (AD) at each terminus and a domain that may be phosphorylated to inhibit transcriptional activation called the inhibitory domain (IHD) (Fig 1.3a) (Berry et al., 2002; Murphy et al., 2004). FOXC1 is a short-lived protein and its expression is tightly regulated (Berry et al., 2006).

1.4.2 *FOXC1 Biological Function*

As a transcription factor, FOXC1 is capable of binding DNA and other proteins to affect transcriptional regulation. FOXC1 incorporation into transcriptional protein complexes or binding DNA targets as a monomer mainly results in transcriptional activation (Berry et al., 2002; Han et al., 2015; Huang et al., 2015; Mirzayans et al., 2012). FOXC1 targets genes involved in various pathways and biological processes. For example, *Msx2* which is an important gene in craniofacial bone development is upregulated when FOXC1 binds its promoter (Mirzayans et al., 2012). FOXC1 also binds the promoters of *CXCR1*, *CC12*, *Snai1*, and *NEDD9* to increase hepatocyte cancer metastasis, inflammation and migration (Hayashi & Kume, 2008; Huang et al., 2015; Xia et al., 2013). Finally, FOXC1 has been found to bind upstream of genes involved in the cell cycle, homeostasis, TGF-β and BMP signalling, metabolism, eye development and intraocular pressure regulation (Berry et al., 2008; Tamimi et al., 2004).

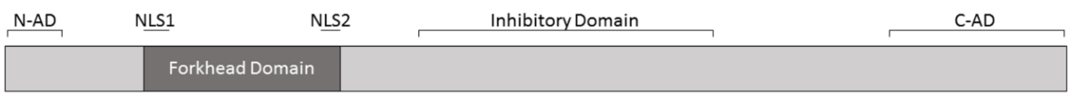
In addition to transactivation of direct targets, FOXC1 is known to bind other transcription factors to affect expression of their or FOXC's targets. Gli2, when bound by FOXC1, increases transcriptional activity of one target: *FAM38B* to induce Hh signalling in Basal-like breast cancer (BLBC) (Han et al., 2015), as well as induces Ihh signalling during endochondral ossification (Yoshida et al., 2015). FOXC1 and PITX2 interact to reduce FOXC1 activity and ensure proper eye development (Berry et al., 2006). Finally, it has been shown that FOXC1 is capable of binding SMAD-4, though it is not known what impact this interaction may have on BMP-SMAD signalling (Fujita et al., 2006).

Figure 1.3

FOXC1 structure and DNA binding. FOXC1 is capable of entering the nucleus and binding DNA. A. FOXC1 contains a transcriptional activation domain at each terminus, an IHD which can be phosphorylated to inhibit FOXC1 activity, and an FHD, which houses a NLS at each domain terminus (Berry et al., 2002). B. FOXC1 FHD is capable of binding DNA through α-helix 3 and wing 2 (Murphy et al., 2004). FOXC1 FHD model based on FOXA3 homology used with permission by the corresponding author.

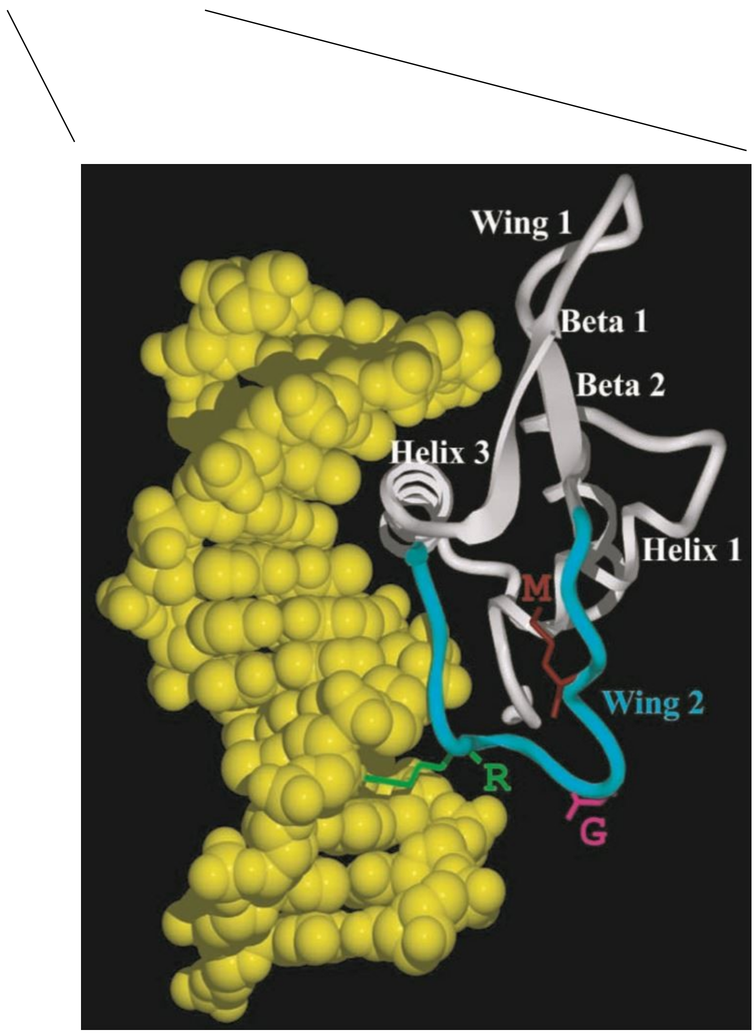
Figure 1.3

A.



N-AD
NLS1
NLS2
Inhibitory Domain
C-AD
Forkhead Domain

B.



Wing 1
Beta 1
Beta 2
Helix 3
Helix 1
M
Wing 2
R
G

FOXC1 has been shown to play key roles in kidney, heart, skeletal, vascular, brain and ocular development (Aldinger et al., 2009; French et al., 2014; Kume et al., 1998; Kume et al., 2000; Mears et al., 1998; Mirzayans et al., 2000; Seo et al., 2006; Swiderski et al., 1999). *FOXC1* expression has also been linked to prognosis in various forms of cancer (Ray et al., 2010; Wang et al., 2016c; Wei et al., 2013; Yu et al., 2015). Increased *FOXC1* expression is linked to a poor prognosis in BLBC, the result of increased epidermal growth factor receptor (EGFR) production driving *FOXC1* expression and downstream targets in the tumor (Jin et al., 2014; Ray et al., 2010; Wang et al., 2012). *FOXC1* upregulation increases cellular migration, invasion and proliferation in BLBC tumors; further, FOXC1 detection in these tumors may become a standard for BLBC diagnosis in the coming years (Jensen et al., 2015; Ray et al., 2010; Wang et al., 2012).

1.4.3 *Mutations to FOXC1*

Mutations to *FOXC1* were first identified in 1998 in connection with eye defects in humans and eye, skeletal and heart anomalies in mice (Kume et al., 1998; Nishimura et al., 1998). Mutations to *FOXC1* may affect protein structure and cellular function. When mutations change amino acid sequence in each NLS, FOXC1 shows reduced ability to enter the nucleus and remains in the cytoplasm until degradation (Berry et al., 2002; Saleem et al., 2003). Mutations affecting the N-terminal end of the FHD can disrupt hydrophobic intramolecular interactions which changes protein conformation enough to reduce DNA binding affinity (Saleem et al., 2003). Mutations to either α-helix 3 or R169 in wing 2 can also reduce DNA binding affinity by affecting direct contact with DNA (Murphy et al., 2004; Saleem et al., 2001). Mutations affecting N- or C-terminal ends of FOXC1 have also been shown to impact transcriptional activation of FOXC1 independent of its DNA binding capacity (Berry et al., 2002; Medina-Trillo et al., 2015). This suggests three things: FOXC1 may in some cases impact transcription of cofactor targets through protein-protein interactions instead of binding DNA, FOXC1 may bind euchromatin improving recruitment of transcriptional machinery and other proteins, and FOXC1 may function as a pioneer factor by binding heterochromatin and making the DNA more available for transcriptional machinery (Iwafuchi-Doi & Zaret, 2014).

Changes in FOXC1 cellular function may result in disease. Loss of or mutations to *FOXC1* have been linked to several disease phenotypes in humans and mice. FOXC1 is truncated by a nonsense mutation to generate *congenital hydrocephalus* (ch) mice (Kume et al., 1998). ch mice have anomalies in several different biological systems including kidneys, meninges, and formation of the eye and skeleton. Mutation to this same region of *FOXC1* is also linked with Axenfeld-Rieger malformations in the anterior region of the eye and early-onset glaucoma, as is duplication to *FOXC1* (Kume et al., 1998; Lehmann et al., 2000; Mears et al., 1998; Mirzayans et al., 2000; Nishimura et al., 2001). FOXC1's role in eye development has been extensively researched and characterized, with mutations to the Forkhead Domain, N-terminal AD (N-AD), the C-terminal AD (C-AD), and the IHD all linked with eye malformation and hypertelorism (Tumer & Bach-Holm, 2009). Deletion to and duplication of chromosomal region 6p25.3 including *FOXC1* is associated with Dandy-Walker Syndrome with cerebellar and eye malformations (Aldinger et al., 2009).

1.5 FOX Genes and Osteogenesis

1.5.1 FOX and Bone Formation

FOXO1 promotes bone formation and binds to *Runx2*'s promoter as osteoblast differentiation proceeds (Siqueira et al., 2011). FOXO1 activity on the promoter is necessary for efficient osteoblast differentiation; FOXO1 knockdown results in lower levels of osteoblast marker expression, ECM gene expression, and mineralization compared to controls (Siqueira et al., 2011). Recently, FOXP expression during endochondral bone formation was investigated, showing that FOXP proteins inhibit chondrocyte hypertrophy and osteoblast differentiation by interacting with RUNX2 (Zhao et al., 2015). Interestingly, FOXP1 and FOXP2 expression was also reported in the jaw during craniofacial ossification, though its role here was not elucidated (Cesario et al., 2016). FOXL2 is also important in proper bone formation, as *Foxl2* $^{-/-}$ mice have delayed ossification *in utero* and skeletal maturation post birth (Marongiu et al., 2015).

Finally, FOXC2 induces paraxial mesoderm differentiation during mesodermal patterning which in turn leads to the formation of the axial skeleton among other tissues (Wilm et al., 2004; Winnier et al., 1997). FOXC2 is required for efficient osteoblast transdifferentiation under BMP-2 induction in C2C12 cells (Yang et al., 2000). *Foxc2* expression is also increased during BMP-4 and -7 induction of limb bud mesenchymal cells, suggesting FOXC2 functions to encourage bone formation in the appendicular skeleton (Nifuji et al., 2001). In addition, *Foxc2* null mice show defects in axial skeletogenesis and FOXC2 knockdown reduces and overexpression increases calvarial bone development through mesenchymal suture maturation, but does not impact calvarial intramembranous ossification (Kim et al., 2009; Rice et al., 2003). Interestingly, FOXC2 aids osteoblast differentiation by activating canonical Wnt signalling and augmenting

BMP signalling (Kim et al., 2009; Yang et al., 2000). Thus, several FOX proteins play important roles in bone development and patterning throughout the body.

1.5.2 *FOXC1 and Bone Formation*

As stated earlier, FOXC1 contributes to skeletal development. *Foxc1* ablation in mice results in malformations of endochondral and intramembranous bone as well as deregulation of gene patterning during somitogenesis (Inman et al., 2013; Kume et al., 1998; Kume et al., 2001; Rice et al., 2003). Endochondral bones like the ribs and digits are smaller and malformed in these mice, and some intramembranous bone such as parietal, interparietal and frontal bones of the skull vault fail to develop at all (Kume et al., 1998). In addition to malformation in the ribs, the spacing between the ribs and axial symmetry is affected, suggesting *Foxc1* plays a role not only in osteogenesis but also proper patterning of bone. *Foxc1*$^{-/-}$ mice develop a fused jaw (syngnathia) resulting from dysregulated neural crest patterning during osteogenic differentiation despite chondrogenesis occurring normally (Inman et al., 2013).

FOXC1's effect on gene expression can change depending on developmental context. FOXC1 interacts with a binding site in the *Msx2* promoter region in C2C12 cells, increasing its expression in response to BMP-2 and inducing *Runx2* expression driving osteoblast differentiation (Mirzayans et al., 2012; Rice et al., 2003; Wilkie et al., 2000). However, this relationship changes depending on location and time during skeletal development. FOXC1 binding to a BMP response element (BRE) in the *Msx2* promoter in calvarial frontal bone reduces BRE availability to BMP-activated SMAD-1/5/8, reducing *Msx2* expression (Sun et al., 2013). Not only does FOXC1 change gene expression in a context-dependent manner, changes in expression are observed from different cells types undergoing osteoblast trans/differentiation. In

C2C12 myoblasts undergoing BMP-induced osteoblast transdifferentiation, *Foxc1* expression increases as differentiation proceeds, and knockdown of FOXC1 results in inefficient BMP-induced differentiation (Hopkins et al., 2015). However, when preosteoblasts are treated with BMP-2/4 to induce differentiation, *Foxc1* expression decreases over time. Similarly, *Foxc1* expression was reduced in response to BMP-2/4 induction in 10T1/2 fibroblasts, MC3T3 preosteoblasts, and D1ORL bone marrow mesenchymal stem cells.

FOXC1 may interact with other proteins to regulate bone development. For example, FOXC1 interacts with Gli2 to increase activation of IHH signalling and ensure proper endochondral ossification (Yoshida et al., 2015). Also, in GST-pull down assays FOXC1 is capable of binding SMAD-4, an important binding partner of pSMAD-1/5/8 in BMP-2/4 induced osteoblast differentiation (Fujita et al., 2006). This interaction which has not been investigated in the literature may have a significant effect on BMP-SMAD signalling and inform us further of the role FOXC1 plays in skeletogenesis.

1.5.3 FOX *and* TGF-β-SMAD *Signalling*

There is extensive evidence that FOX proteins play crucial roles in TGF-β and BMP signalling. FOXL2 FHD interacts with SMAD-3 MH1 domain to induce expression of *Follistatin*, an antagonist of many TGF-β proteins (Blount et al., 2009). FOXL2 also induces cartilage and skeletal development with SOX9, perhaps under TGF-β induction (Marongiu et al., 2015). Also, FOXOs 1, 3 and 4 are capable of binding SMAD-3 and SMAD-4 through FHD to MH1 domains respectively to activate *p21Cip1* in response to TGF-β (Seoane et al., 2004). Also, as stated earlier, FOXO1 is induced by BMP-2 and binds the *Runx2* promoter late in MC3T3 preosteoblast differentiation (Siqueira et al., 2011). SMAD-2 and SMAD-4 each bind to FOXH1 to bind specific target genes in response to TGF-β induced expression of activin (Chen et al., 1996; Liu et al., 1997). Similarly, FOXH1 was recently shown to repress the Nodal pathway as a mechanism of regulation (Nodal is a member of the TGF-β family) (Reid et al., 2016). Activated Smad-2 binds FoxH1 and separates FoxH1 from Nodal repressor TLE4, reducing Nodal repression and allowing proper mesoderm formation. However, FOXG1 inhibits both general and FOXH1-activated gene expression under TGF-β induction by binding SMAD-2 and preventing SMAD-2 interaction with FOXH1 at DNA targets (Dou et al., 2000; Rodriguez et al., 2001). FOXF1 regulates vascular development and formation by inducing *Bmp-4* expression (Astorga & Carlsson, 2007; Mahlapuu et al., 2001). *Foxf1* expression is important for Bmp-4 mediated growth of the mesodermal lateral plate as *Foxf1* null mutants show less *Bmp-4* expression and reduced proliferation of the lateral mesoderm and intestine (Mahlapuu et al., 2001; Ormestad et al., 2006). FOXM1 also directly interacts with TGF-β activated SMAD-3 to induce SLUG expression and promote cancer metastasis (Xue et al., 2014). Finally, FOXP3 is likely not directly bound by SMADs induced by TGF-β or BMP, but its expression in T-cells is induced through the TGF-β signalling pathway (Grainger et al., 2010; Jana et al., 2009).

1.5.4 *FOXC and TGF-β/BMP Signalling in Development*

Like other FOX proteins, FOXC2 also interacts with TGF-β and BMP signalling directly and indirectly to regulate gene expression. FOXC2 outcompetes FOXO1 suppression of insulin- and TGF-β-activated PAI-1 promoter by directly binding SMADs 3 and 4 and synergistically activating PAI-1 (Fujita et al., 2006). FOXC1 and FOXC2 activate transcription of the Notch ligand *Dll4* during lymphatic and vascular development (Seo et al., 2006). Because there is significant crosstalk and interaction between TGF-β and Notch signalling throughout the developing body, FOXC activation of Notch signalling may impact TGF-β and BMP signalling during development (Blokzijl et al., 2003; Dahlqvist et al., 2003). BMP signalling induces transcription of FOXC1 and FOXC2 in mouse eyelid development (Huang et al., 2009). FOXC2 is also required for and is sufficient to stimulate osteoblast differentiation in MC3T3 cells (Kim et al., 2009). Despite the fact that BMP-2/4 induction is also sufficient to stimulate osteoblast differentiation, Kim et al. did not investigate the relationship between BMP signalling and *Foxc2* expression in preosteoblasts (2009). However, because BMP-2/4 can induce Foxc2 expression elsewhere during development such as limb mesenchyme and mouse mesodermal cells (Nifuji et al., 2001), FOXC2 may physically interact with BMP signalling to induce osteoblast differentiation in MC3T3 cells as well.

TGF-β and BMP-SMAD signalling and FOXC1 activity overlap in many biological systems. BMP signalling is important in angiogenesis and induces heart progenitor cells to undergo differentiation into cardiomyocytes, and FOXC1 is important in the early development of the heart and blood vasculature (Boyd et al., 2007; Kume et al., 2001; Tirosh-Finkel et al., 2010). BMP-2/4 and FOXC1 are both important in proper kidney development (Carev et al., 2008; Kume et al., 2000). *Foxc1* expression is upregulated by IL-8, which is stimulated by TGF-β, to induce metastasis in cancer (Huang et al., 2015; Lu & Dong, 2006). Vertebral mesoderm requires FOXC1 and FOXC2 for paraxial cell fate determination and BMP-2 treatment favors intermediate and lateral patterning early in development (James & Schultheiss, 2005; Wilm et al., 2004).

BMP-4 induced development of chondrogenic nodules in condensing mesenchyme is FOXC1-dependent (Kume et al., 1998). It is also suggested that FOXC1 is induced by fibroblast growth factor (FGF) signalling to act synergistically with BMP signalling in calvarial bone development (Rice et al., 2005). FOXC1 can bind TGF-β activated SMAD-3 as well as SMAD-

4; the latter being common to both TGF-β and BMP signalling (Fujita et al., 2006). Similarly, FOXC1 directly binds and increases activity of HH transcription factor Gli2 which is also capable of being activated by TGF-β signalling (Dennler et al., 2007; Han et al., 2015). FOXC1 downregulates BMP-induced *Msx2* expression in calvarial bones (Sun et al., 2013). FOXC1 can also bind BMP2K, which is induced by BMP-2 treatment (Tamimi et al., 2004). Finally, FOXC1 is upregulated when BMP-4 induces C2C12 cells to differentiate to osteoblasts and stable, ectopic FOXC1 expression increases Alpl production similar to BMP-4 treatment in C2C12 cells (Hopkins et al., 2015; Mirzayans et al., 2012).

1.6 Rationale and Hypothesis

In summary, FOX proteins interact with, regulate and are regulated by TGF-β and BMP signalling in many aspects of biology from development to homeostasis and cancer growth (Blount et al., 2009; Chen et al., 1996; Cui et al., 2005; Huang et al., 2015; Lu & Dong, 2006). FOXC1 is important in mesoderm patterning, eye formation, renal and cardiac development, vascularization, and both endochondral and intramembranous ossification (French et al., 2014; Han et al., 2015; Inman et al., 2013; Kume et al., 1998; Kume et al., 2000). TGF-β and BMP signalling overlap and interact with FOXC1 to regulate many of these systems. It is clear that spatial and temporal regulation of *FOXC1* expression is crucial to proper ossification in different bones at specific times (Hopkins et al., 2015; Mirzayans et al., 2012; Sun et al., 2013). However, the relationship between FOXC1 and BMP signalling in developing bone is not fully understood. Therefore, I hypothesize that FOXC1 interacts with BMP signalling to ensure proper bone patterning and development.

To understand the role FOXC1 plays in BMP signalling four aims were investigated. The first aim was to determine if FOXC1 influences SMAD activation of Id1 BRE under BMP induction. The second aim was to identify where FOXC1 interacts in the BMP signalling cascade. The third aim was to identify what regions of FOXC1 are necessary for its activity on BMP signalling. Finally, the fourth aim was to determine endogenous cellular responses to *FOXC1* overexpression in cells capable of osteoblast differentiation.

Chapter Two: Materials and Methods

2.1 Tissue Culture

U2OS (human osteosarcoma) and C2C12 (mouse myoblast) cells were grown in 10mL Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MI Cat. # D6429) supplemented with 10% Fetal Bovine Serum (FBS) in a T-75 cell culture flask incubated at 37˚C, 5% CO_2 until 85-95% confluent. The media was then aspirated and the cells washed with 10mL 1x Phosphate-buffered Saline (PBS), and treated with 1mL Trypsin-ethylenediamine tetra-acetic acid (EDTA) (TE) Buffer to dislodge them from the surface of the flask. Once the cells were coated with TE, the remaining buffer was aspirated and cells placed in the incubator for 1-2 minutes. The cells were then collected from the flask surface by rinsing with 10mL 10% FBS in DMEM. Finally, 1-2mL of the cells in medium were transferred to a new flask with media and incubated. The remaining cells in suspension were counted by hemocytometer for use in experiments described below.

2.2 Transactivating Assays

24-well plates were seeded with U2OS or C2C12 cells at a density of $4x10^4$ cells/mL in DMEM + 10% FBS to a total volume of 1 mL per well and incubated at 37˚C, 5% CO_2. The cells reach approximately 60% confluence after 24 hours of incubation. The cells were transfected with 150ng/mL of each affector plasmid (FOXC1 wildtype (WT) or deletion/mutation constructs, empty vector (EV) or SMADs), 10-100ng/mL of reporter plasmid (BRE-luc), 0.1ng/mL of Renilla plasmid, and 3:1 volume:mass ratio of Mirus *Trans*IT®-LT1 transfection reagent (Mirus Bio LLC, Madison WI) to DNA. FOXC1-S131L and deletion constructs were created previously (Berry et al., 2002; Saleem et al., 2003). Reagents were added to 50µL DMEM/well and incubated undisturbed in a fume hood for 30 minutes. The mixture was mixed by pipetting, centrifuged, and equal parts of the mix was added to each appropriate well in the 24-well plate and incubated for 24 hours before recombinant human BMP-4 (rhBMP-4) (R&D Systems, Minneapolis, MN) treatment, or 48 hours if untreated. Assays underwent at least three technical replicates for each of three biological replicates.

To treat the cells with BMP-4, the cell media was aspirated then rinsed with 1mL/well of 1x PBS twice. DMEM + 10% FBS with either 50ng/mL BMP-4 or equivalent volume of 4mM HCl (solution BMP-4 is diluted in when preparing aliquots) was added to each appropriate well to a total volume of 1mL/well. 24 hours after treatment, the cells were washed three times in 1x PBS before cellular lysis using 100µL/well of 1x Passive Lysis Buffer (Dual-Luciferase® Reporter Assay, Promega, Madison WI) at room temperature while rocking gently for 20 minutes. Luciferase Assay is as described in the Dual-Luciferase® Reporter Assay protocol (Promega, WI) using 95µL of LARII and STOP'n'GLO (Promega). Statistical analysis was carried out using One-way ANOVA tests with Holm-Sidak post hoc and correction (Figs 3.1-3.2), Kruskal-Wallis One-way ANOVA on Ranks and Dunn's tests (Fig 3.4) because U2OS had unequal columns. Additional testing on C2C12 luciferase (Fig 3.4b) was carried out using Tukey's tests with no changes in significance observed from Dunn's test.

2.3 Protein Isolation

U2OS or C2C12 cells were cultured in the conditions outlined in Table 2.1. Media was aspirated from the cells, which were then washed twice in 5 or 10mL 1x PBS or Tris-buffered Saline (TBS) for 60mm or 100mm plates, respectively. 1.3 or 5mL of 1x PBS was then added

Table 2.1: Cell Culturing Conditions for Proteins

Cell Type	U2OS		C2C12	C2C12/pBABE/ *FOXC1*
Plate Size	60 mm	100 mm	60 mm	100 mm
Cell Density	$5x10^5$ cells/plate	$1x10^6$ cells/plate	$5x10^5$ cells/plate	$1x10^6$ cells/plate
Duration of Growth	72 hours	72 hours	72 hours	48 hours
Transfection	2 µL plasmid at 24 hours of growth	4 µL plasmid at 24 hours of growth	2 µL plasmid at 24 hours of growth	No
3:1 Mirus:DNA	Yes	Yes	Yes	N/A
BMP-4 Treatment	50 ng/mL - 250 ng total	50 ng/mL - 500 ng total	50 ng/mL - 250 ng total	50 ng/mL - 500 ng total

to 60mm or 100mm plates respectively. The cells were then scraped into 1.7mL microcentrifuge tubes or 15mL falcon tubes and centrifuged at 2500rpm 4˚C for 10 minutes to pellet the cells. The PBS was aspirated and the pellet resuspended 1:1 in a Cell Lysis Buffer made up of the following: 20mM Hepes pH 7.6, 20% glycerol, 10mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.1% TritonX-100, 1mM DDT, 1mM PMSF, 0.5% protease inhibitor cocktail (PIC). The cells were then sonicated for 3 second bursts at 30% intensity 3 times using a Biodisrupter followed by 30-60 minutes mixing at 4˚C under gentle rotation. The mixture was then centrifuged at high speed (25000rpm 15 minutes 4˚C) to pellet cellular debris. The supernatant containing proteins was transferred to a new microcentrifuge tube for quantification. Quantification was carried out using a Bradford Assay (Bio-Rad) with 0, 2, 5, 10, 15, and 20µg/µL Bovine serum albumin (BSA) standards.

2.4 Western Blotting

Protein was prepared for Western Botting (WB) by dilution in 12.5µL standard 2x sodium dodecyl sulphate (SDS) to 25µL total volume for each lane to be loaded (50µL for two lanes, etc.). ddH_2O was added to each mix to reach the necessary volume. The master mixes were incubated at 95˚C for 5 minutes before being loaded onto 10-15% polyacrylamide gels for protein size separation. The proteins were then transferred onto a nitrocellulose membrane for 1 hour at 350mA or overnight at 30mA at 4˚C. The membrane was blocked using 5mL Licor Odyssey Blocking Buffer (PBS) (Licor Biosciences, Lincoln, NB) at room temperature for 1 hour under gentle rocking. Primary antibody was added to 2.5mL of each Tris-buffered Saline plus 0.05% Tween 2.0 (TBST) and Blocking Buffer for 1 hour at room temperature or overnight at 4˚C under gentle rocking. Primary antibody dilutions are listed in Table 2.2. Six 5 minute washes in TBST at room temperature removed residual primary antibody before the appropriate secondary antibody Donkey anti-Rabbit, Donkey anti-Mouse, or Donkey anti-Goat (Life Technologies, Bengaluru, India) was diluted 1:10000 in 2.5mL each TBST and Blocking Buffer and added to the membrane. Secondary antibody treatment proceeded for 1 hour at room temperature under gentle rocking. Six 5x TBST washes removed residual secondary antibody, and the membrane was emerged in TBS briefly until imaged using Licor Odyssey Infrared Imager (Licor).

2.5 Differentiation Assays

2.5.1 *Cell Culturing*

For 24-hour differentiation assays 6-well plates or 35mm plates were seeded with C2C12, C2C12-pBABE, or C2C12-pBABE-*FOXC1* at a density of $2x10^5$ cells/well in 2mL DMEM + 10% FBS (Mirzayans et al., 2012). For the six-day differentiation assays 6-well plates were seeded in duplicate (one plate for RNA extraction, one plate for alkaline phosphatase (ALP) staining) with C2C12-pBABE or C2C12-pBABE-*FOXC1* at an initially density of $2x10^5$ cells/well in 2mL DMEM + 10% FBS. After 48 hours' incubation at 37˚C, 5% CO_2, the cells were aspirated, washed once with 1x PBS, and treated with either 50ng/mL BMP-4 or equivalent volume of 4mM HCl in 2mL serum-reduced media (DMEM + 0.2% FBS). For the 24-hour assays, the cells were grown in low serum media and BMP or mock for 24 hours. For the 6-day assays the cells either immediately underwent RNA extraction and ALP staining or placed in the

Table 2.2: Antibodies for Western Blotting

1˚ Antibody	Company	Catalog Number		Dilution
Goat anti-FOXC1 C-18	Santa Cruz Biotech	SC-21396	polyclonal	1:500
Mouse anti-Beta-Tubulin	Santa Cruz Biotech	SC-55529	monoclonal	1:5000
Mouse anti-FLAG	GeneScript	A00187	monoclonal	1:5000
Mouse anti-FLAG	Sigma-Aldrich	F1804	monoclonal	1:5000
Mouse anti-HA	Santa Cruz Biotech	SC-4633369	polyclonal	1:1000
Mouse anti-SMAD4 B-8	Santa Cruz Biotech	SC-79667	monoclonal	1:1000
Mouse anti-XPRESS	Invitrogen	R910-25	monoclonal	1:5000
Rabbit anti-HA Y-11	Santa Cruz Biotech	SC-805	polyclonal	1:1000
Rabbit anti-Id1	Santa Cruz Biotech	SC-488	polyclonal	1:500
Rabbit anti-pSMAD 1/5/8	Cell Signalling	9511	polyclonal	1:1000
Rabbit anti-pSMAD 1/5/9	Cell Signalling	D5B10	monoclonal	1:1000
Rabbit anti-SMAD5	Cell Signalling	D4G8	monoclonal	1:1000

incubator. Cells were incubated for 0, 24, 96, or 144 hours before undergoing RNA extraction or ALP staining (Figure 2.1).

2.5.2 *RNA Extraction*

Media was aspirated from the cells before washing twice in 2 mL 1x PBS. RNA extraction was carried out using the RNeasy Plus Mini Kit Animal Cell Spin technique (Cat# 74106 Qiagen, Mississauga, Canada) using the protocol provided. RNA was then quantified using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA)

2.5.3 *ALPL Stain*

Media was aspirated from the cells before washing twice in 2 mL 1x PBS. Cells were fixed in 1.5 mL/well formalin (3.7% formaldehyde in 1x PBS pH 7.4) for 20 minutes. Cells were then washed twice in 2 mL 1x PBS and stained in 1 mL BCIP/NBT liquid substrate system (Sigma-Aldrich) in the dark (covered with foil) for 3.5 hours. Staining solution was removed and the cells rinsed extensively in ddH_2O before being imaged by confocal microscopy. Two images per well of each replicate plate were taken. Each image was quantified using percent of area stained using ImageJ software. Percent staining outliers were removed and difference was tested with a one-tailed t-test for equal columns or Mann-Whitney Rank Sum test for unequal columns of data.

2.5.4 *Qualitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)*

QuantiTect Reverse Transcriptase Kit (Qiagen Cat. # 205313) was used to create cDNA from 500 ng of RNA. 13 µL of 1:25 diluted cDNA was added to 16.25 µL KAPA 2x SYBR Fast Master Mix (KAPA Biosystems, Wilmington, MA) and 3.25 µL primer sets (Table 2.3) to create the master mix. The master mix was then vortexed briefly, spun down and 10 µL placed into each of three wells in a 96-well plate for technical replicates. Once loaded, the plate was sealed and centrifuged briefly before qPCR was carried out using the following protocol: 95˚C 3 minutes, (95˚C 5 seconds, 60˚C 20 seconds) x 40 cycles, followed by a melting curve from 65˚C-95˚C in 0.5˚C increments for 5 seconds each on a BIO-RAD CFX96 Touch real time PCR detection system. Data was analyzed using Bio-Rad CFX Manager version 3.0.125.0601 normalized to three housekeeping genes: *Gapdh, Hprt,* and *Actin B*.

Figure 2.1

Differentiation Time Course Schematic. Cells were cultured for 24 hours before changing media to low serum (0.2% FBS) with BMP-4 or mock treatment. Cells used for Day 0 Alpl staining were not treated with BMP-4. Immediately following BMP-4 or mock treatment, RNA was isolated from Day 0 cells or returned to the incubator. Cells were cultured for 1, 4, or 6 days before Alpl staining or RNA isolation.

Figure 2.1

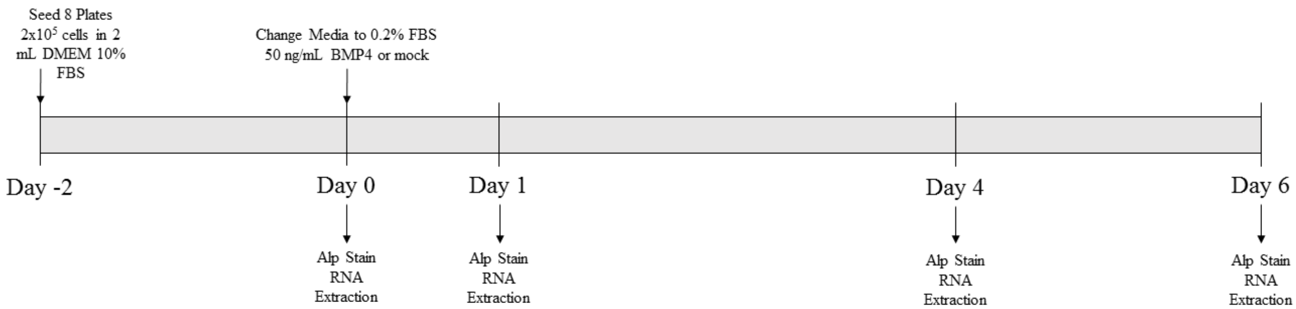

Seed 8 Plates
$2x10^5$ cells in 2 mL DMEM 10% FBS
Change Media to 0.2% FBS
50 ng/mL BMP4 or mock
Day -2
Day 0
Day 1
Day 4
Day 6
Alp Stain
RNA Extraction
Alp Stain
RNA Extraction
Alp Stain
RNA Extraction
Alp Stain
RNA Extraction

Table 2.3: QRT-PCR Primers

Primer Name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
msFoxc1	CCCTGCTTATTGTCCCGATAG	GCTACATCGCTCTTATCACCA
hsFoxc1 Set 2	TGTTCGAGTCACAGAGGATCG	ACAGTCGTAGACGAAAGCTCC
hsFoxc1 Set 3	AGAACTTCCACTCGGTGCG	CCCGTTCACTGGAGAGTTGT
msId1	CGACTACATCAGGGACCTGCA	GAACACATGCCGCCTCGG
msRunx2	ACCATGGTGGAGATCATCG	TAACAGCGGAGGCATTTCG
msOsx	CTTCTTTGTGCCTCCTTTCC	GCGTCCTCTCTGCTTGA
msSpp1	TCGTCATCATCGTCGTCCA	AGAATGCTGTGTCCTCTGAAG
msAlpl	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
msActinb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
msGapdh	GTGGAGTCATACTGGAACATGTAG	AATGGTGAAGGTCGGTGTG
msDlx3	IDT PrimeTime Sequence 141824991	
msBmp2k	Rewference Sequence NM_080708(1)	
msFgfr1	IDT PrimeTime Sequence 138800169	
mfFgfr2	AATCTCCCAACCAGAAGCGTA	CTCCCCAATAAGCACTGTCCT
msMsx2	TTCACCACATCCCAGCTTCTA	TTGCAGTCTTTTCGCCTTAGC
msHey1	IDT PrimeTime Sequence 139854873	
msCol1a1	AGGGCCAAGACGAAGACATC	AGATCACGTCATCGCACAACA
msHprt	AACAAAGTCTGGCCTGTATCC	CCCCAAAATGGTTAAGGTTGC

Chapter Three: Results

3.1 FOXC1 Impact on BMP-induced BRE Activation

FOXC1 is known to play an important role in ossification and BMP-induced chondrogenesis (Kume et al., 1998). Previous research from Hopkins et al demonstrated that FOXC1 in addition to being upregulated by BMP-4 is required for efficient BMP-induced osteogenesis (Hopkins et al., 2015). My initial aim was to determine if increased levels of FOXC1 enhances BMP-signalling. I made use of luciferase transactivation assays carried out in U2OS (human osteosarcoma) cells. U2OS cells are a good model for this system because they are of an osteogenic lineage and are easily transfected. This assay models BMP-signalling by insertion of 2 copies of the *Id1* BRE enhancer into the promoter region driving luciferase expression. Endogenously, BRE is bound by BMP-activated SMAD complex, driving *Id1* expression (Katagiri et al., 2002). This reporter is used to monitor BMP signalling both *in vitro* and *in vivo* (Collery & Link, 2011).

BMP-4 treatment increases luciferase expression approximately 13-fold over mock treatment in cells transfected with an XPRESS (XP)-tagged pcDNA4.0 empty vector (EV) (Figure 3.1). When XP-FOXC1 is exogenously expressed in these cells BMP-induced BRE activation is greatly reduced. Interestingly, un-induced cells express less luciferase when XP-FOXC1 is present compared to EV. Because FOXC1 and FOXC2 are from the same subfamily of FOX proteins, and share close FHD homology, I decided to exogenously express XP-FOXC2 in U2OS cells and repeat the assay (Kaestner et al., 2000). BRE activation is also reduced in FOXC2 expressed cells. This tells us there may be some functional overlap or redundancy between FOXC1 and FOXC2 that produces this reduction in BRE activation.

3.2 FOXC1 impact on SMAD-induced BRE activation

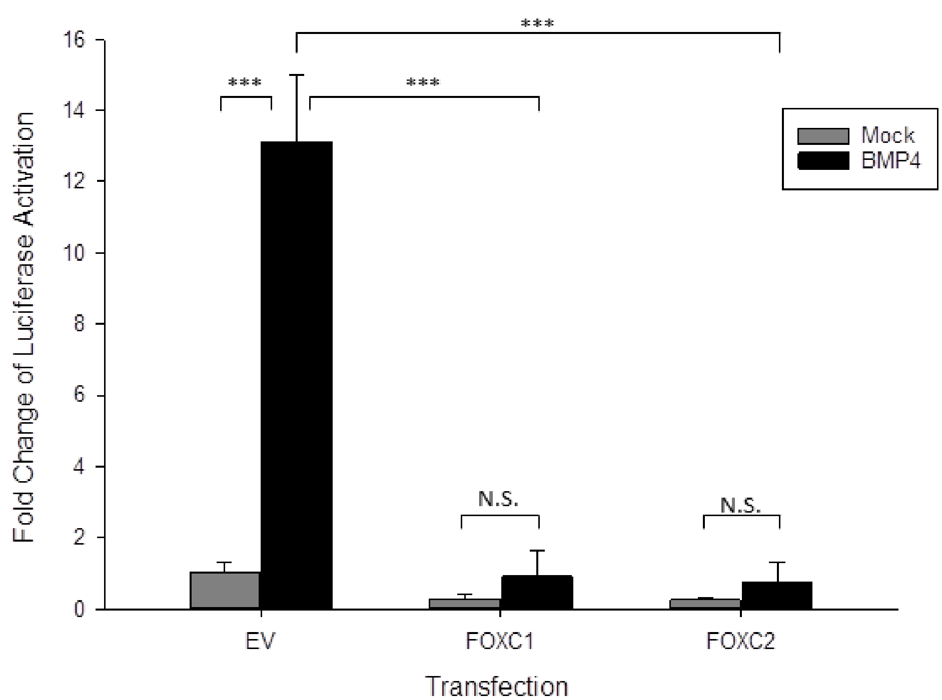
Next I wanted to determine at what point in the BMP-SMAD signalling pathway FOXC1 interacts. FOXC1 may interact with BMP antagonist Noggin, with BMPRs, SMADs, SMAD complex cofactors, or at the DNA level to inhibit BMP signalling. Using the same assay as before, I co-transfected cells with either three empty vectors (pcDNA4.0-XP, pcI-HA, pFLAG), XP-FOXC1 with two empty vectors, HA-SMAD5 and FLAG-SMAD4 and XP-EV, or XP-FOXC1, FLAG-SMAD-4 and HA-SMAD-5. SMAD transfection is capable of BRE reporter activation *in vitro* (Hopkins et al., 2015), and the resulting impact FOXC1 has on this activation will inform our understanding of FOXC1's interaction in BMP signalling. If SMAD-activated BRE is not reduced in the presence of FOXC1 its interaction is dependent on BMP activation of the signalling pathway and FOXC1 may interact with BMPRs or Noggin. If FOXC1 decreases SMAD-activated BRE, FOXC1 interacts downstream of SMAD activation at the protein or DNA level of BMP-SMAD signalling.

Preliminary data of SMADs 4 & 5 co-transfection results in increased BRE activation of luciferase expression in the absence of BMP-4. This means that SMADs 4 & 5 together in high quantity are able to mimic BMP-4 treatment without the need for BMP receptor activation by BMP-4 dimers (Figure 3.2). If FOXC1 still reduces BRE activation levels without ligand and receptor involvement in the experiment, I can limit my hypothesis of FOXC1 activity on BRE activation to protein-protein or protein-DNA interaction. When FOXC1 is exogenously expressed with SMADs 4 & 5, BRE activation is reduced relative to SMAD 4 & 5. This shows that FOXC1 interacts with other proteins like SMADs or accessory proteins, or DNA targets to reduce BRE activation by SMADs.

Figure 3.1

FOXC1 and FOXC2 reduce BRE activation. U2OS cells transfected with XP-EV, XP-FOXC1, or XP-FOXC2 plasmids. Four biological replicates had three technical replicates per assay. Statistical analysis was carried out using One-way ANOVA with Holm-Sidak test and correction. Asterisks represent statistical significance. ***: $P < 0.001$.

Figure 3.1

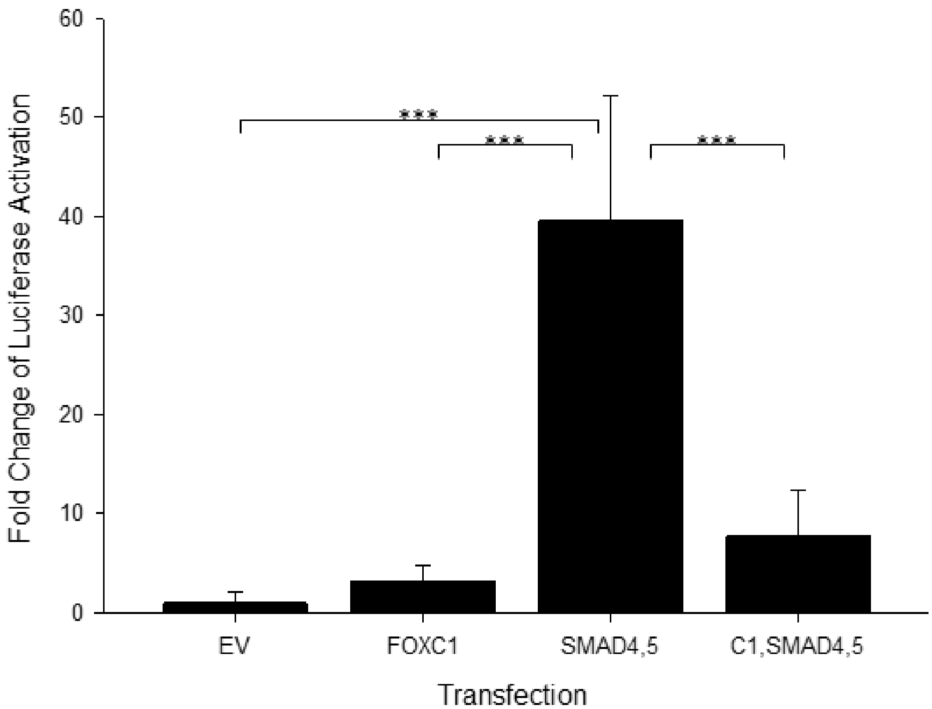


***
***
***
Mock
BMP4
N.S.
N.S.
Fold Change of Luciferase Activation
16
14
12
10
8
6
4
2
0
EV
FOXC1
FOXC2
Transfection

Figure 3.2

FOXC1 reduces SMAD-induced BRE activation. Preliminary luciferase assay data. U2OS cells were transfected with: XP-EV or XP-FOXC1, pCI-HA or HA-SMAD-5, and pFLAG or FLAG-SMAD-4. One biological replicate was technically replicated three times. Statistical analysis was carried out using a One-way ANOVA test and Holm-Sidak correction. Asterisks represent statistical significance. ***: $P < 0.001$.

Figure 3.2


60
50
40
30
20
10
0
Fold Change of Luciferase Activation
***
***
***
EV
FOXC1
SMAD4,5
C1,SMAD4,5
Transfection

3.3 FOXC1 functional region participation in BRE activity reduction

Because FOXC1 reduces BRE activation, and does so by interacting with either proteins or DNA, I wanted to determine if specific regions of FOXC1 were necessary for its inhibitory function on BMP signalling. To do this, I made use of deletion constructs previously created (Figure 3.3A) (Berry et al., 2002). These constructs have either the N-AD missing, the C-AD deleted, the IHD-C-term region deleted, or only the FHD present (Fig 3.3A). When the N-AD is deleted from FOXC1, it no longer reduces BMP-induced BRE activation. When the C-AD is deleted, levels are lower than EV transfected cells, though significantly different than WT levels of BRE activation. Constructs missing the IHD show increased levels of BRE activation both with and without BMP-4 treatment beyond those seen in the EV control. There is no difference between the two treatments, suggesting that BMP-4 treatment no longer activates BRE in the presence of this construct. Finally, when only the FHD is present, basal BRE levels are increased as with the IHD-C-term deletion, though BMP-4 treatment results in a further increase in activation. These results show there is functional importance to the N-AD and the IHD in how FOXC1 reduces BRE-activation, as all constructs are expressed in the cells (Figure 3.3B). In addition, SMAD activation is not affected by FOXC1 presence in the cells, as cells transfected with each construct and treated with BMP-4 are still displaying phosphorylated SMAD1/5/8 at similar levels to EV+BMP-4 (Figure 3.3C).

3.3.1 ***Evaluation of DNA-binding function in*** **BMP*-induced BRE activation***

Next, I sought to discover if DNA-binding function was necessary for my finding that FOXC1 reduces BRE activation with and without BMP induction. A plasmid containing a FOXC1 Serine 131 to Leucine mutation which is able to translocate to the nucleus, but unable to bind DNA was used (Saleem et al., 2001). C2C12 as well as U2OS cells were transfected because C2C12 cells are capable of differentiating to osteoblasts when treated with BMP-4. When FOXC1 S131L is transfected in U2OS cells, the BRE is no longer reduced when treated with BMP-4 (Figure 3.4A). When C2C12 cells are transfected, there is no significant difference between S131L and WT constructs despite WT FOXC1 reducing BRE activation (Fig 3.4B). However, there is also no difference between S131L and EV transfections in this cell line. FOXC1-S131L is strongly expressed in U2OS cells (Fig 3.4C) suggesting the loss of BRE activation reduction is due to the loss in DNA-binding ability.

3.4 *FOXC1* overexpression impact on BMP-induced C2C12 cells

3.4.1 *Evaluation of ectopic FOXC1 expression impact on endogenous* BMP-SMAD *pathway gene expression after 24 hours of BMP-4 treatment*

Increased FOXC1 production in U2OS cells reduces BMP stimulated BRE activation. To determine if this occurs endogenously in BMP-induced cells, the endogenous expression of BMP-induced genes when *FOXC1* is overexpressed was evaluated. C2C12 cells containing a stably-expressing *FOXC1* gene were used (Mirzayans et al., 2012). WT C2C12 cells, pBABE empty vector lentiviral control cells (C2C12-pBABE), and FOXC1 cells (C2C12-pBABE-*FOXC1*) were initially tested together to determine if increased *FOXC1* expression affects BMP-induced gene expression and if C2C12-pBABE cells are a suitable control for subsequent qPCR experiments. Increased FOXC1 production was indicated by Western Blot (Figure 3.5A), and that this increase is the result of *FOXC1* expression (Fig 3.5B). Interestingly, there was an

Figure 3.3

FOXC1 N-AD, IHD and FHD are important in BRE reduction. **A.** Luciferase assay of deletion construct activity on BRE reporter. U2OS cells were transfected with: XP-EV, XP-FOXC1, XP-FOXC1 29-553, XP-1-366, XP-1-215, or XP-FOXC1 75-268. **B.** Expression analysis of FOXC1 deletion constructs. ⊦: band at expected size of deletion construct. **C.** SMAD phosphorylation in *FOXC1*-overexpressing cells. Cells were cultured and treated as in B. Proteins were detected using anti-pSMAD 1/5/8 primary antibody and anti-Tubulin B primary antibody. Protein map of FOXC1 is modified from Berry et al figure 6 (2002). Statistical analysis was carried out using Kruskal-Wallis One-Way ANOVA on Ranks and pairwise Student-Newman-Keuls comparison. Asterisks represent statistical significance. *: $P < 0.05$ vs EV + BMP-4; †: $P < 0.05$ vs WT + BMP-4.

Figure 3.3

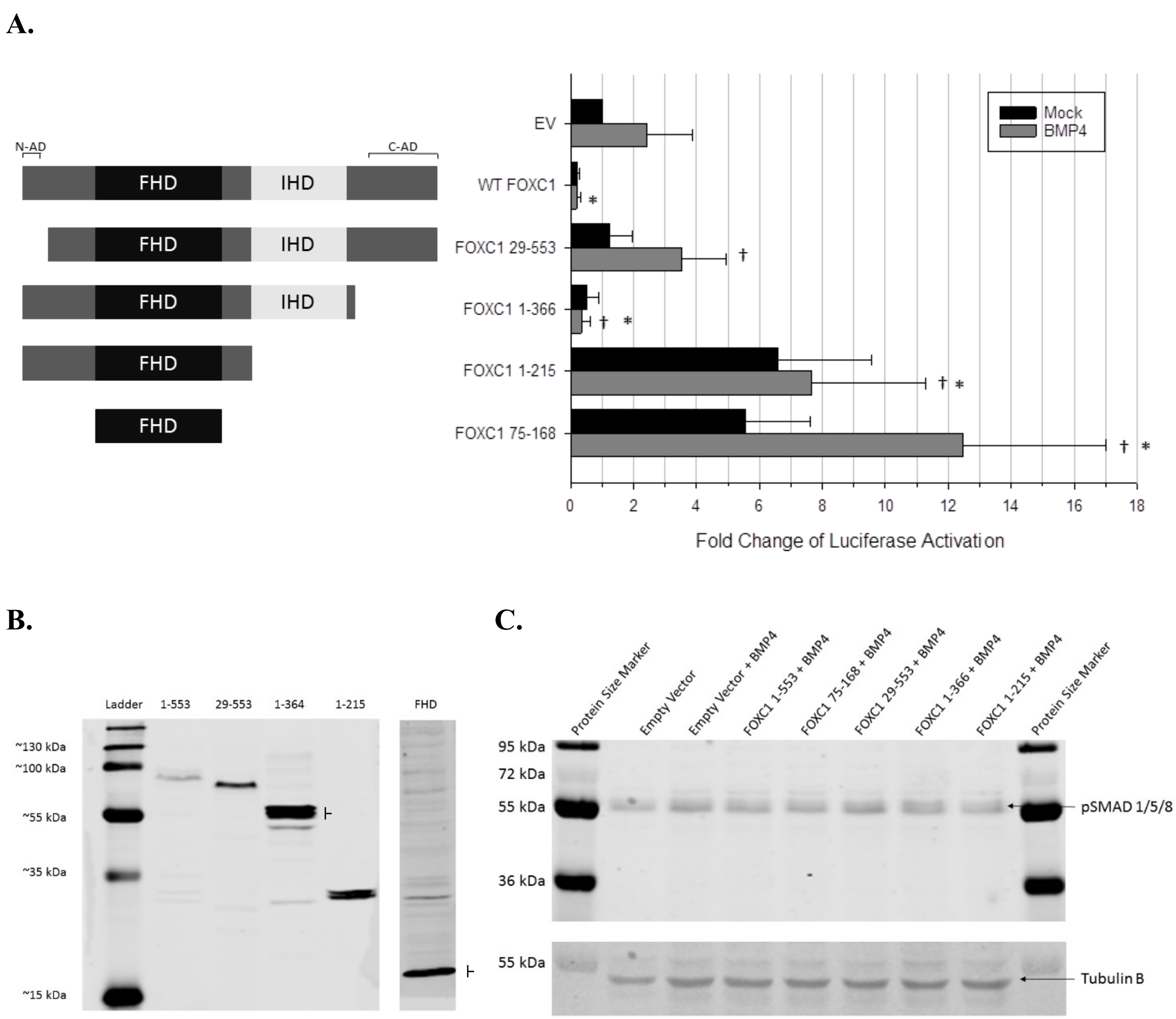

A.
N-AD
C-AD
FHD
IHD
EV
WT FOXC1
FOXC1 29-553
FOXC1 1-366
FOXC1 1-215
FOXC1 75-168
Mock
BMP4
0 2 4 6 8 10 12 14 16 18
Fold Change of Luciferase Activation
B.
Ladder
1-553
29-553
1-364
1-215
FHD
~130 kDa
~100 kDa
~55 kDa
~35 kDa
~15 kDa
C.
Protein Size Marker
Empty Vector
Empty Vector + BMP4
FOXC1 1-553 + BMP4
FOXC1 75-168 + BMP4
FOXC1 29-553 + BMP4
FOXC1 1-366 + BMP4
FOXC1 1-215 + BMP4
Protein Size Marker
95 kDa
72 kDa
55 kDa
36 kDa
pSMAD 1/5/8
55 kDa
Tubulin B

Figure 3.4

Functional FHD is required for FOXC1-mediated reduction of BRE. U2OS **(A)** and C2C12 **(B)** cells were transfected with XP-EV, XP-FOXC1 or XP-FOXC1 S131L. **C.** U2OS cells were transfected with XP-EV, XP-FOXC1 or XP-FOXC1 S131L. FOXC1 protein was detected using anti-XP antibody. Three biological replicates had three technical replicates per assay. Statistical analysis was carried out for both U2OS and C2C12 cells using Kruskal-Wallis One-way ANOVA on Ranks and Dunn's tests because U2OS had unequal columns. Additional testing on C2C12 luciferase was carried out using Tukey's tests with no changes in significance observed from Dunn's test. Asterisks represent statistical significance. N.S.: not significant; *: $P < 0.05$; **: $P < 0.01$.

Figure 3.4

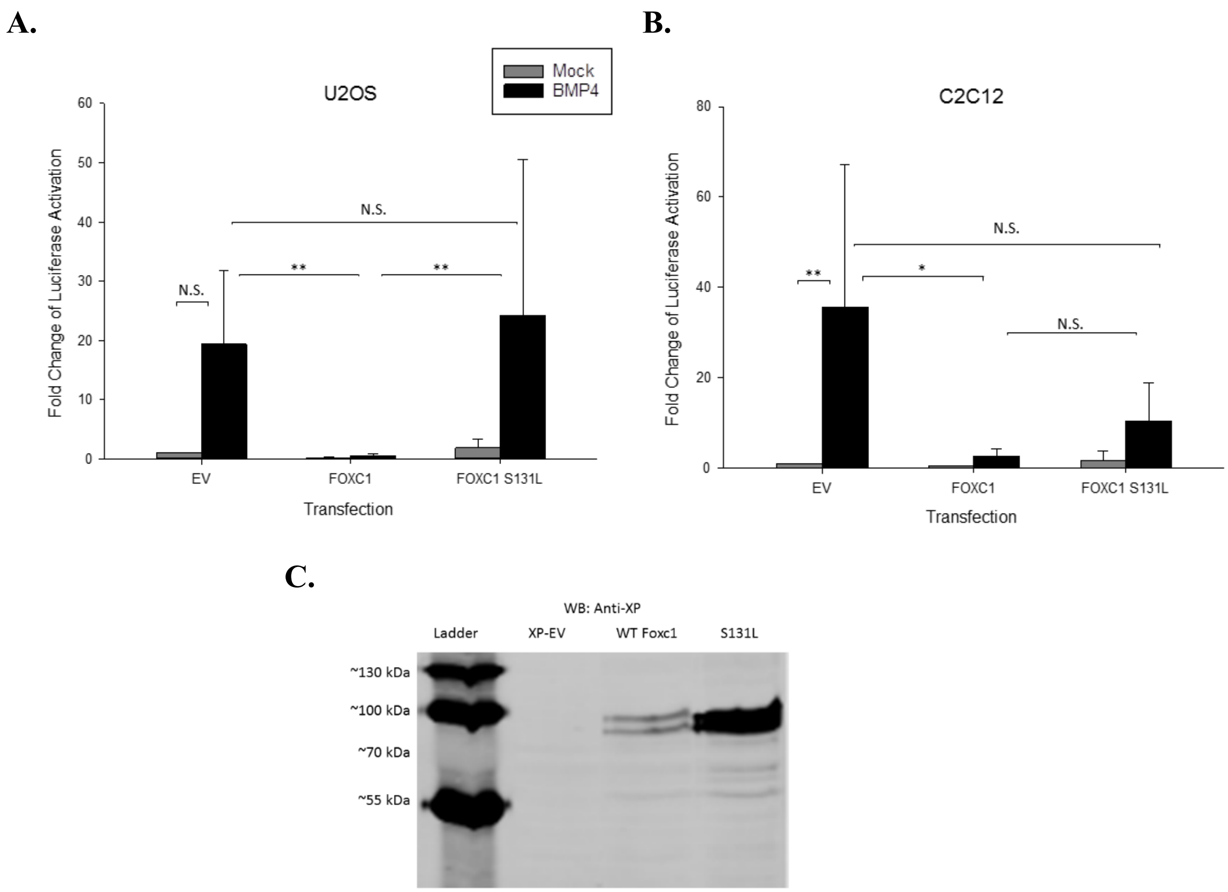
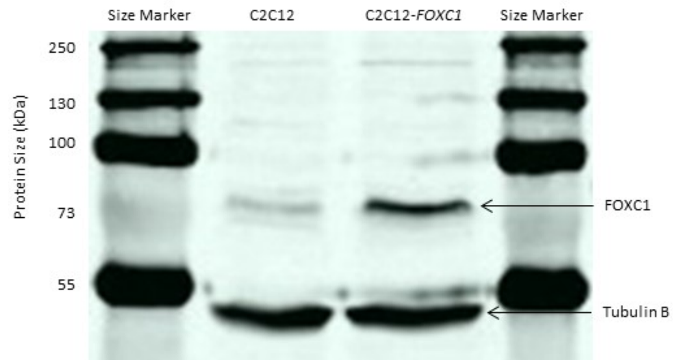

A.
U2OS
Mock
BMP4
Fold Change of Luciferase Activation
0
10
20
30
40
50
60
N.S.
**
**
N.S.
EV
FOXC1
FOXC1 S131L
Transfection
B.
C2C12
0
20
40
60
80
**
*
N.S.
N.S.
EV
FOXC1
FOXC1 S131L
Transfection
C.
WB: Anti-XP
Ladder
XP-EV
WT Foxc1
S131L
~130 kDa
~100 kDa
~70 kDa
~55 kDa

Figure 3.5

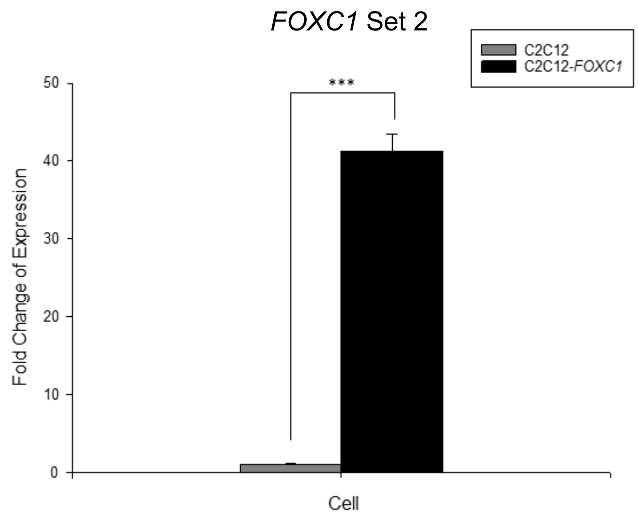
C2C12-pBABE-*FOXC1* cells express human and mouse *FOXC1*. **A.** C2C12-pBABE-*FOXC1* protein expression analysis. Anti-FOXC1 primary antibody was used to detect FOXC1 protein in C2C12 and C2C12-pBABE-*FOXC1* cells. **B.** qPCR amplifying human *FOXC1* cDNA. **C.** qPCR amplifying mouse *Foxc1* cDNA. Significance was tested using CFX Manager proprietary software. Asterisks represent statistical significance. **: $P < 0.01$; ***: $P < 0.001$.

Figure 3.5

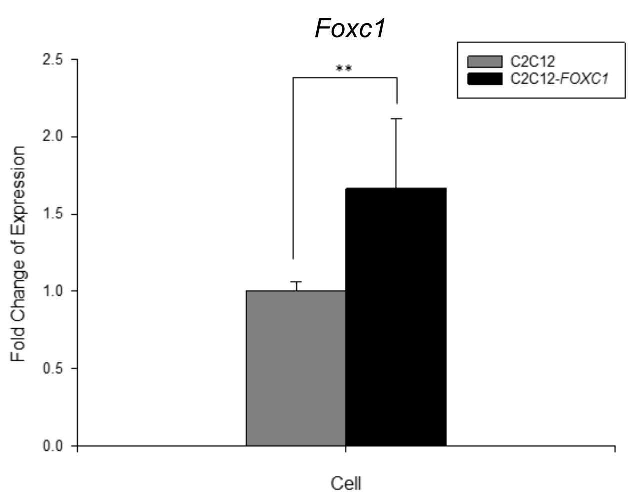
A.



B.



C.



increased level of *Foxc1* expression, indicated by qPCR (Fig 3.5C). This is likely due to the primers for *Foxc1* amplifying both human and mouse *FOXC1* transcripts.

Because *Id1* BRE was used in the luciferase experiments, *Id1* expression was the initial focus in this experiment. BRE response to BMP induction was reduced in those experiments, which led to the hypothesis that *Id1* expression will be reduced under BMP induction when *FOXC1* is overexpressed. Figure 3.6 shows BMP treatment increases *Id1* expression in all three cells. There is no difference between C2C12 and C2C12-pBABE cells in untreated and BMP-treated *Id1* expression. However, both the wildtype and control cells differ from C2C12-pBABE-*FOXC1*. C2C12-pBABE-*FOXC1* cells have reduced basal levels of *Id1* expression as well as when treated with BMP, although BMP treatment does increase *Id1* expression in these cells to the level observed in other cell types (Hopkins et al., 2015; Mirzayans et al., 2012).

Next, other genes known to be upregulated by BMP treatment were evaluated to determine whether FOXC1 acts on BMP signalling globally or on specific targets such as *Id1*. *Runx2* is well-characterized to be induced by BMP and is upregulated during osteoblast differentiation (Lee et al., 2000). Although *Runx2* production is not increased in C2C12 cells in the 24-hour treatment (Figure 3.7A), C2C12-pBABE cells show increased *Runx2* production when treated with BMP-4. Interestingly, basal levels of *Runx2* in C2C12-pBABE-*FOXC1* cells are increased compared to pBABE controls, and under induction by BMP-4, increase further compared to both C2C12 and C2C12-pBABE.

Another gene induced by BMP-4 in C2C12 cells is *Hey1* (Zamurovic et al., 2004). *Hey1* is a canonical Notch pathway gene, but is upregulated under BMP induction to interact with

Runx2 and control cellular differentiation (Dahlqvist et al., 2003; Zamurovic et al., 2004). Here, *Hey1* is upregulated by BMP-4 treatment in all cell lines (Fig 3.7B). However, basal expression in C2C12-pBABE and C2C12-pBABE-*FOXC1* cells is lower than wildtype cells. Any differences in BMP-induced expression of *Hey1* in these three cells do not reach significance.

3.4.2 *Evaluation of ectopic* FOXC1 *overexpression impact on endogenous osteoblast marker gene expression after 24 hours of* BMP-4 *treatment*

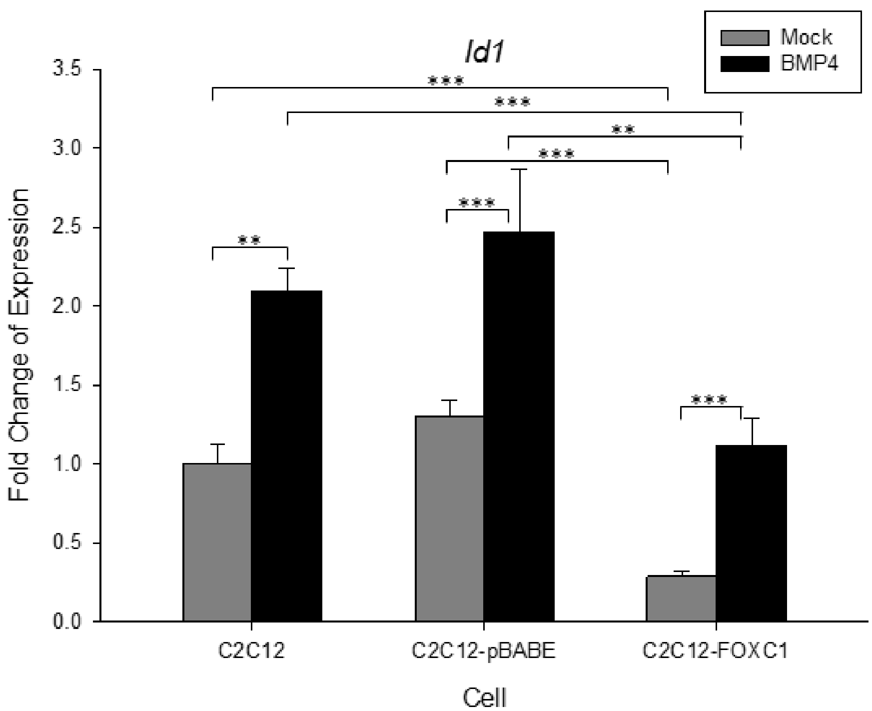
In addition to examining the above genes in the 24-hour assay, osteoblast markers were also amplified to determine if *FOXC1* overexpression results in changes to osteoblast differentiation in C2C12 cells. *Alpl* is expressed and distributed extracellularly early in osteogenesis and histochemical staining of Alpl is a common technique to determine osteoblast differentiation in cells (Lorch, 1949; Mirzayans et al., 2012). Here, *Alpl* expression dramatically increases in *FOXC1* overexpressing cells compared to C2C12 and C2C12-pBABE cells (Fig 3.8A). There is no significant difference in the expression profile between C2C12 and C2C12-pBABE cells. Interestingly, BMP treatment results in reduced levels of *Alpl* expression in C2C12-pBABE-*FOXC1* cells compared to untreated cells.

Next, *Col1a1* expression was evaluated (Fig 3.8B). Col1a1 is a subunit of the protein type 1 collagen that makes up much of the trabecular meshwork in bone and is expressed through osteoblast differentiation and maturation (Beck et al., 2001; Bedalov et al., 1995). Despite no upregulation of *Col1a1* in C2C12 cells in this experiment, C2C12-pBABE cells show increased *Col1a1* expression when treated with BMP-4 (Fig 3.8B). Both treated and untreated C2C12-pBABE-*FOXC1* cells have increased *Col1a1* expression, though BMP treatment does not increase expression over basal levels here.

Figure 3.6

FOXC1 overexpression reduces *Id1* expression. *FOXC1* overexpression effect on *Id1* promoter in C2C12, C2C12-pBABE or C2C12-pBABE-*FOXC1* cells. The experiment was repeated three times with three technical replicates per plate. Data was analyzed using CFX Manager software. Asterisks represent statistical significance. **: $P < 0.01$; ***: $P < 0.001$.

Figure 3.6



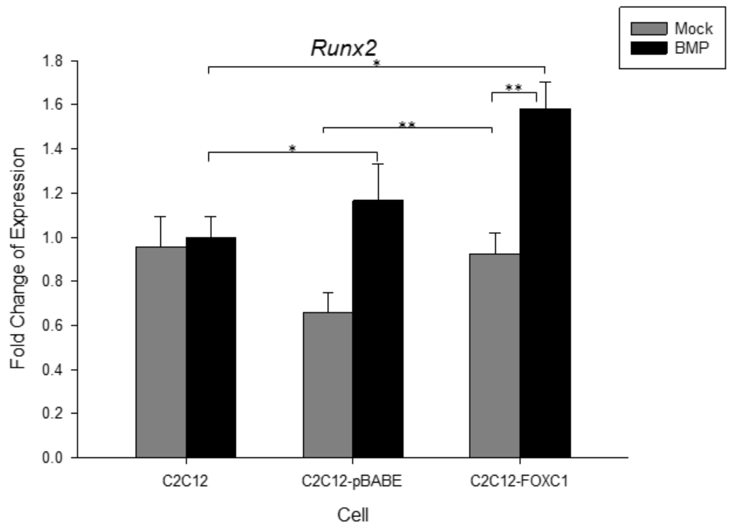
Id1
Mock
BMP4
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fold Change of Expression
***
***
**
***
***
**
***
C2C12
C2C12-pBABE
C2C12-FOXC1
Cell

Figure 3.7

FOXC1 overexpression does not immediately affect *Runx2* or *Hey1* expression. *Runx2* **(A)** and *Hey1* **(B)** transcripts were amplified by qPCR. The experiment consisted of three technical replicates per plate, and was repeated 3 times. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Comparisons not described are not statistically significant.

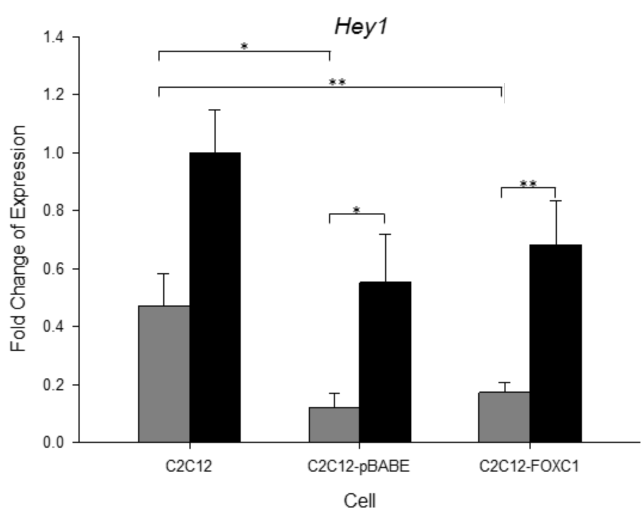
Figure 3.7

A.



Runx2
Mock
BMP
Fold Change of Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C2C12
C2C12-pBABE
C2C12-FOXC1
Cell

B.



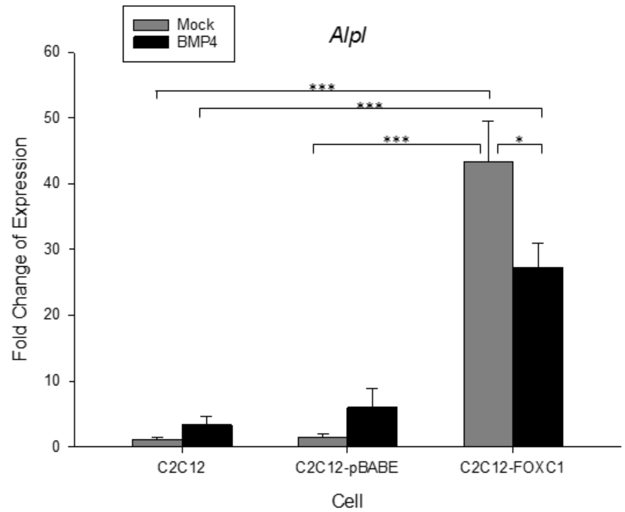
Hey1
Fold Change of Expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C2C12
C2C12-pBABE
C2C12-FOXC1
Cell

Figure 3.8

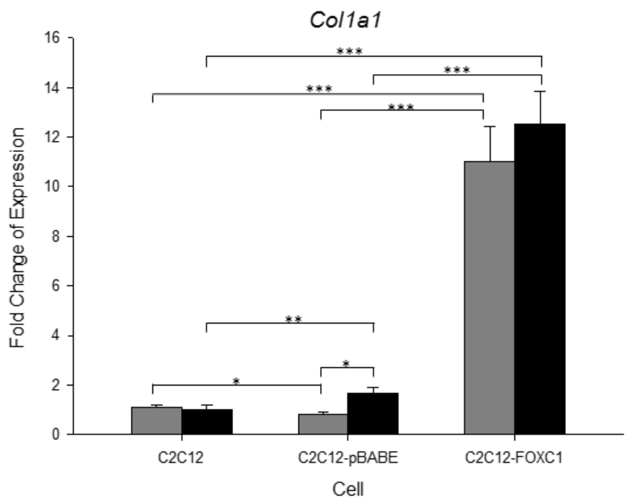
Ectopic *FOXC1* expression affects *Alpl* and *Col1a1* expression. *Alpl* **(A)**, *Col1a1* **(B)**, and *Osx* **(C)** transcripts were amplified by qPCR. Experiment was replicated three times with three technical replicates per PCR plate. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Comparisons not described are not statistically significant.

Figure 3.8

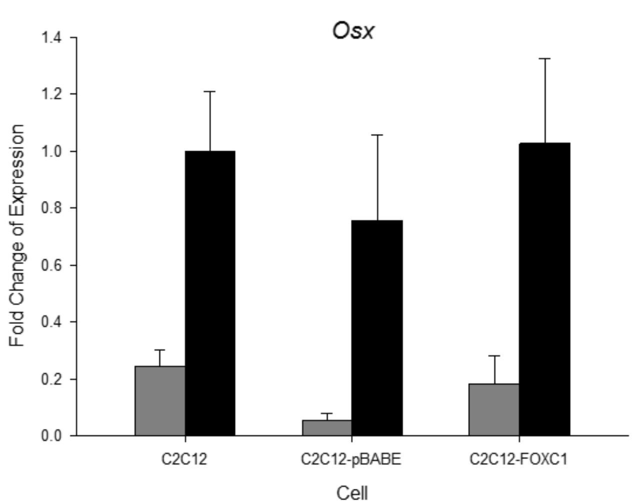
A.



B.



C.



Osx is another gene upregulated during osteoblast differentiation (Nakashima et al., 2002; Nishio et al., 2006). It is a late marker for osteoblast differentiation (Lee et al., 2003). In this experiment, *Osx* expression in the three cell types did not reach levels required for statistical analysis (Fig 3.8C).

3.4.3 FOXC1 *overexpression impact on expression of other* BMP*-induced genes*

In addition to the BMP-SMAD genes and osteoblast markers examined, *Bmp2K (Bmp2 inducible kinase*), *Fgfr1* and *Fgfr2* (*fibroblast growth factor receptorss*) were evaluated (Fig 3.9). *Bmp2k* is upregulated by BMP-2/4 and its stable expression results in reduced ALPL and *Ocn* levels independent of *Runx2* expression (Kearns et al., 2001). FOXC1 is capable of binding the *Bmp2k* promoter (Tamimi et al., 2004); thus overexpression may change the expression pattern of *Bmp2k* in these cells. Untreated C2C12-pBABE-*FOXC1* cells had lower *Bmp2k* expression than WT cells (Figure 3.9A), though no difference was seen in BMP-treated cells. pBABE control cells had increased *Bmp2k* expression with and without BMP treatment.

Fgfr1 is expressed in mesenchymal cells to drive pre-osteoblast differentiation, though expression then declines to allow proper osteoblast maturation (Jacob et al., 2006; Su et al., 2014). It has been shown that *Fgfr1* is not regulated by, neither does it regulate *Foxc1* expression in mouse calvaria (Rice et al., 2005). However, stable *FOXC1* expression may have an impact on *Fgfr1* expression in C2C12 myoblasts. In Fig 3.9B, PCR amplification of *Fgfr1* transcripts shows that untreated C2C12-pBABE-*FOXC1* have lower *Fgfr1* expression compared to controls. BMP-treated cells show no change in expression compared to WT, but less *Fgfr1* expression compared to pBABE controls. *Fgfr2* is also expressed to promote osteoblast differentiation, though predominantly in cranial sutures (Su et al., 2014). *Fgfr2* also differs from *Fgfr1* expression in that it does not inhibit osteoblast maturation. *Fgfr2* expression does not change in calvarial growth of mice lacking *Foxc1* expression (Rice et al., 2005). However, their research does suggest that Fgf2 treatment results in increased *Foxc1* expression during calvarial growth. In cells overexpressing *FOXC1*, *Fgfr2* expression is reduced compared to both control cells regardless of treatment (Fig 3.9C). BMP treatment increases *Fgfr2* expression in both the pBABE control and *FOXC1* overexpressing cells compared to untreated cells.

3.5 Six-day time course evaluating ectopic *FOXC1* expression impact on BMP-SMAD pathway and osteoblast markers

Given that *FOXC1* overexpression altered early osteogenic induction events, we sought to determine what effect *FOXC1* overexpression has in long term osteoblast differentiation. Osteoblast differentiation is a tightly controlled process, and *FOXC1* appears to be one of the genes tightly regulated in this process (Hopkins et al., 2015). Based on the 24-hour qPCR and Alpl staining assays, I hypothesized that *FOXC1* overexpression induces osteoblast differentiation in C2C12 cells while only affecting BMP-SMAD signalling at *Id1* expression. C2C12-pBABE and C2C12-pBABE-*FOXC1* cells were cultured as described above. However, instead of RNA extraction after 24 hours, at each time point (immediately after BMP-4/mock treatment and after 1, 4 and 6 days of low serum culturing with or without BMP treatment) RNA extraction and ALP staining was carried out (Figure 2.1).

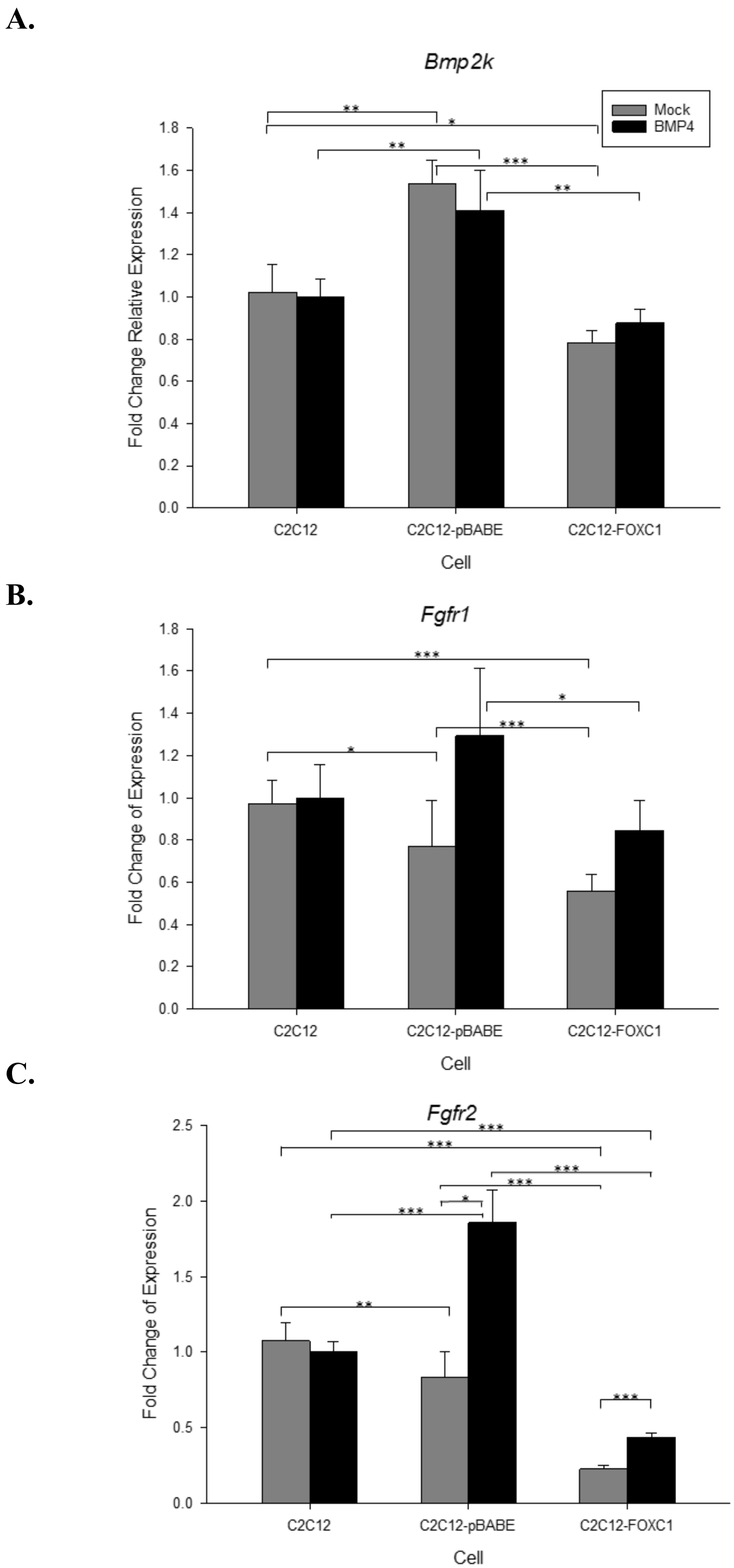
3.5.1 *Ectopic* FOXC1 *expression on long-term* BMP-SMAD *pathway gene expression*

As in the 24-hour qPCR assay, BMP-SMAD genes were examined (Figures 3.10-13). In untreated cells, *FOXC1* overexpression results in decreased levels of *Id1* expression throughout

Figure 3.9

FOXC1 overexpression reduces *Fgfr2* expression. The impact of *FOXC1* overexpression on *Bmp2k* **(A)**, *Fgfr1* **(B)**, and *Fgfr2* **(C)** expression was evaluated. Transcripts were amplified by qPCR. Significance of three biological x three technical replicates was calculated using CFX Manager software. Asterisks represent statistical significance. *: P < 0.05; **: P < 0.01; ***: P < 0.001. Comparisons not described are not statistically significant.

Figure 3.9


A.
Bmp2k
Mock
BMP4
Fold Change Relative Expression
C2C12
C2C12-pBABE
C2C12-FOXC1
Cell
B.
Fgfr1
Fold Change of Expression
C2C12
C2C12-pBABE
C2C12-FOXC1
Cell
C.
Fgfr2
Fold Change of Expression
C2C12
C2C12-pBABE
C2C12-FOXC1
Cell

the time course, though not significantly immediately after mock treatment, compared to pBABE controls (Fig 3.10A). However, when cells are treated with BMP-4, significant decreases in *Id1* expression are seen in C2C12-pBABE-*FOXC1* cells only at Day 0 and Day 6 (Fig 3.10B). There were no significant changes in *Runx2* expression between C2C12-pBABE and C2C12-pBABE-*FOXC1* mock-treated cells (Fig 3.11A). In BMP-4-treated cells, *FOXC1* overexpression reduced *Runx2* expression early in the time course, though only significantly at Day 0 (Fig. 3.11B).

Dlx3 is BMP-SMAD target that is expressed in epidermal cells undergoing differentiation, as well as the perichondrium and chondrocytes during embryonic and vertebral growth (Hassan et al., 2004; Park & Morasso, 2002; Peng et al., 2003). *Dlx3* is activated as early as one hour after BMP induction in C2C12 cells (Hassan et al., 2004). *Dlx3* induces *Ocn* expression at low concentrations by binding its promoter, but is then outcompeted by DLX5. It is hypothesized that DLX3 in high concentrations binds RUNX2, sequestering it from the *Ocn* promoter and reducing *Ocn* expression. Recently, it has been shown that *Dlx3* expression is not affected in *Foxc1*-ablated mice (Inman et al., 2013). In *FOXC1* overexpressing cells, *Dlx3* appears to be upregulated relative to pBABE cells until near the end of the time course in untreated cells, though significance is only reached on Day 1 (Fig 3.12A). When treated with BMP-4, *Dlx3* is also upregulated when *FOXC1* is overexpressed, though here it occurs towards the end of the time course, though only significant at Day 4 (Fig 3.12B). Finally, *Hey1* levels are initially increased in mock-treated C2C12-pBABE-*FOXC1* cells but at days 4 and 6 they are dramatically decreased relative to pBABE control cells (Fig 3.13A). No difference is seen between C2C12-pBABE-*FOXC1* and C2C12-pBABE cells treated with BMP-4, though *Hey1* expression increases in both cells at day 1 of treatment (Fig 3.13B).

3.5.2 FOXC1 *overexpression on BMP-induced osteogenic differentiation*

Alpl expression was then analyzed both by qPCR and ALP staining (Figures 3.14-3.16). In mock-treated C2C12-pBABE-*FOXC1* cells *Alp* expression increases dramatically on days 1, 4 and 6 relative to C2C12-pBABE cells, which show little to no *Alpl* induction throughout the time course (Fig 3.14A). Interestingly, *Alpl* expression is much reduced on day 1 of BMP-4 treatment relative to control, but increases for days 4 and 6 (Fig 3.14B). The spike in expression in the C2C12-pBABE cells returns to basal levels by day 4 and remained low at day 6 (Fig 3.14B). Mock-treated control cells show little difference in Alpl staining from day 1 to day 6 of culturing (Fig 3.15); however, mock-treated C2C12-pBABE-*FOXC1* cells show a dramatic increase in Alpl production as time progresses. C2C12-pBABE cells treated with BMP gradually stain darker for Alpl as time progresses (Fig 3.16). *FOXC1* overexpressing cells treated with BMP-4 produce less Alpl than control cells on day 1 with no difference initially or after day 1(Fig 3.16). Interestingly, mock-treated *FOXC1* overexpressing cells stain darker for Alpl on day 4 compared to BMP-treated cells (Fig 3.15, 3.16, $P < 0.05$).

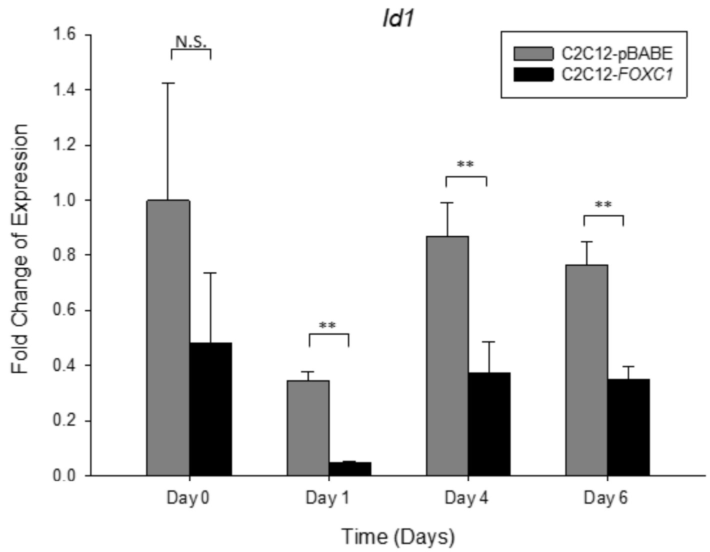
Next, *Col1a1*, *Opn*, and *Osx* expression were observed throughout the time course to further evaluate the effect *FOXC1* overexpression has on osteoblast differentiation (Figures 3.17-3.19). In mock treated cells (Fig 3.17A), *FOXC1* overexpression results in increased levels of *Col1a1* expression throughout the time course, though not significantly at day 0. *Col1a1* gradually increases in control cells under BMP induction (Fig 3.17B), whereas heightened expression begins quickly in C2C12-pBABE-*FOXC1* cells: peaking at day 1 before gradually declining. *Col1a1* expression is higher in C2C12-pBABE-*FOXC1* cells compared to control cells throughout the BMP-4 treatment time course. Interestingly, *Opn* expression declines early in mock-treated C2C12-pBABE-*FOXC1* cells as the time course progresses, whereas control cells

Figure 3.10

FOXC1 overexpression reduces *Id1* expression during osteoblast differentiation. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.10

A.



B.

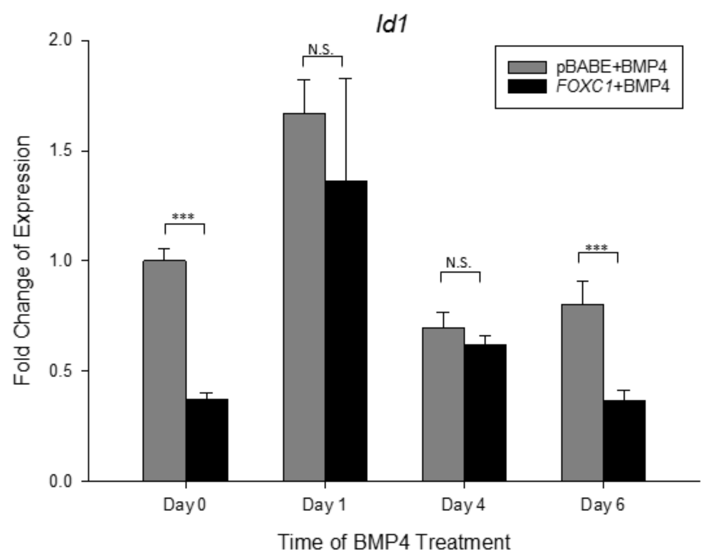
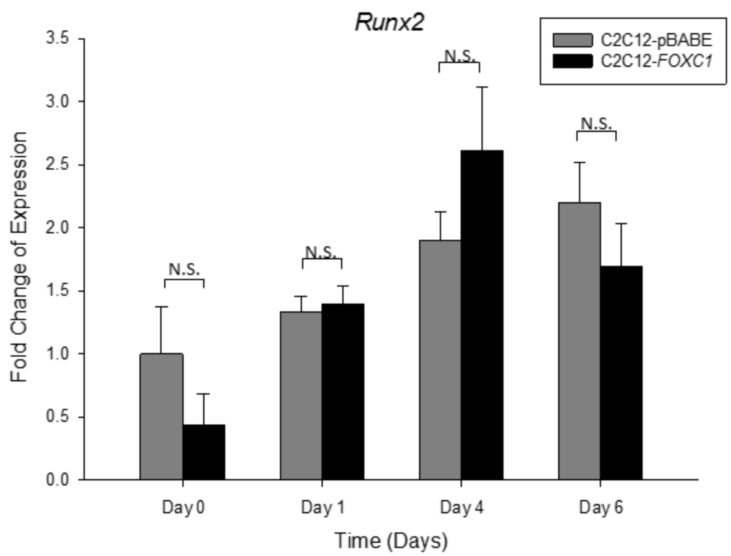


Figure 3.11

FOXC1 overexpression reduces early BMP-4 induced *Runx2* expression. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.11

A.



B.

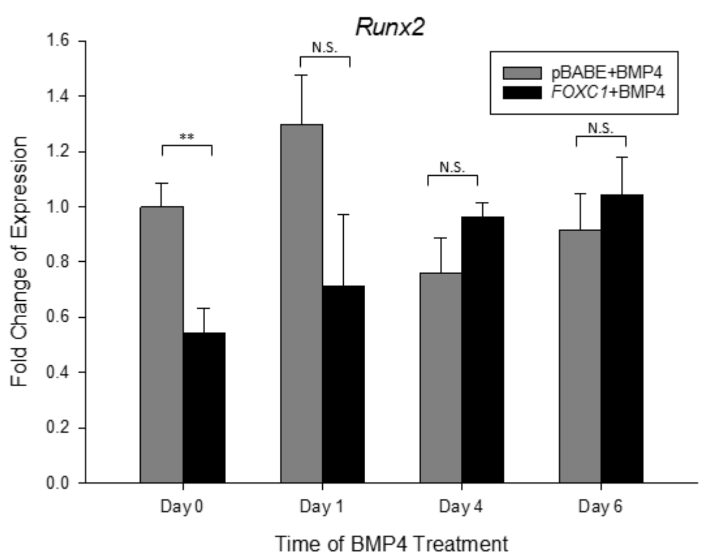
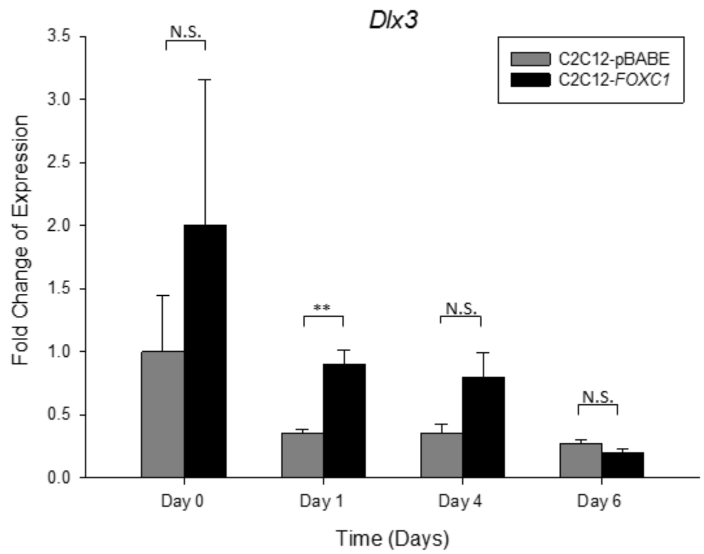


Figure 3.12

FOXC1 overexpression changes *Dlx3* expression during osteoblast differentiation. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.12

A.



B.

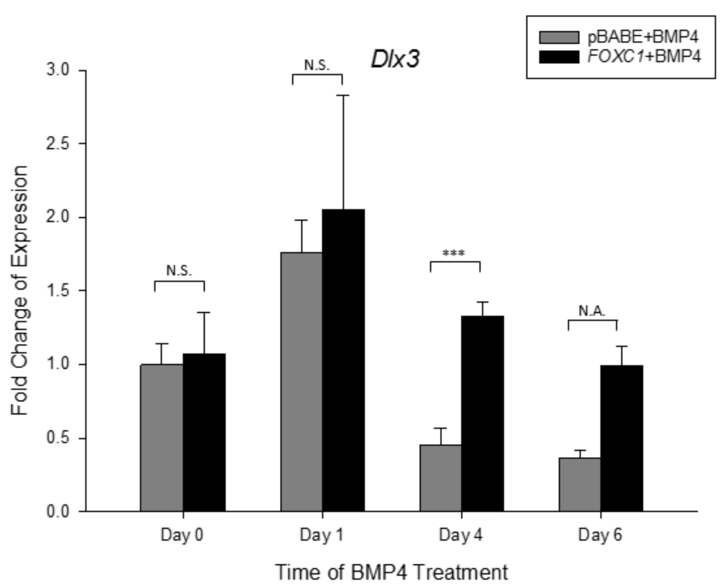
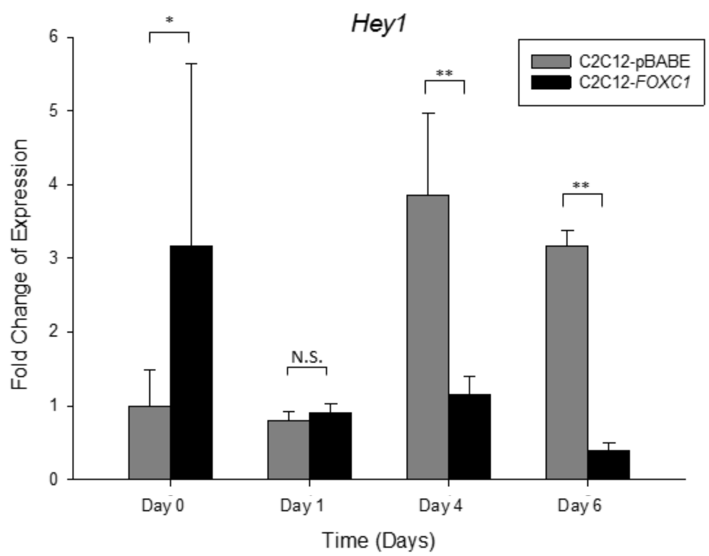


Figure 3.13

FOXC1 overexpression affects *Hey1* expression during ectopic osteoblast differentiation. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.13

A.



B.

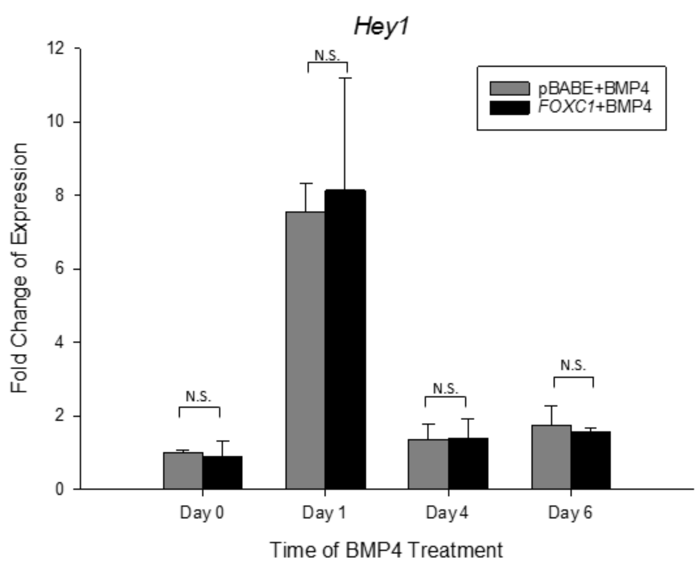
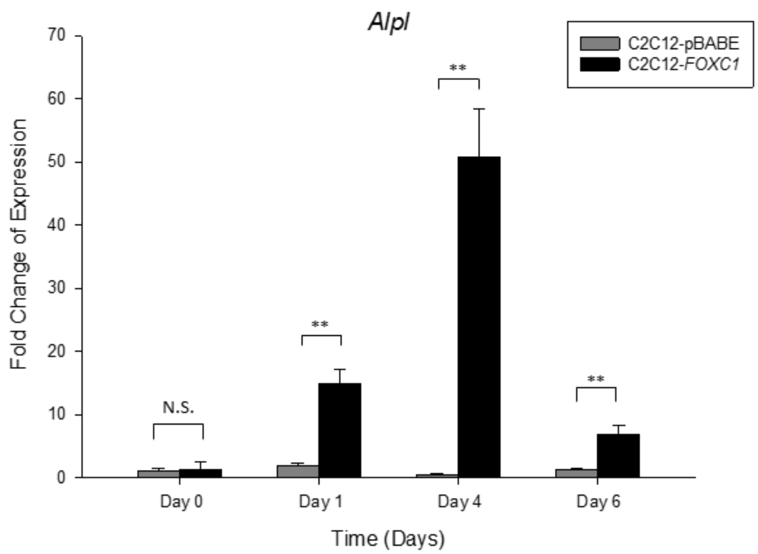


Figure 3.14

FOXC1 overexpression increases *Alpl* expression during ectopic osteoblast differentiation, and delays *Alpl* expression during BMP-4 induced osteoblast differentiation. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.14

A.



B.

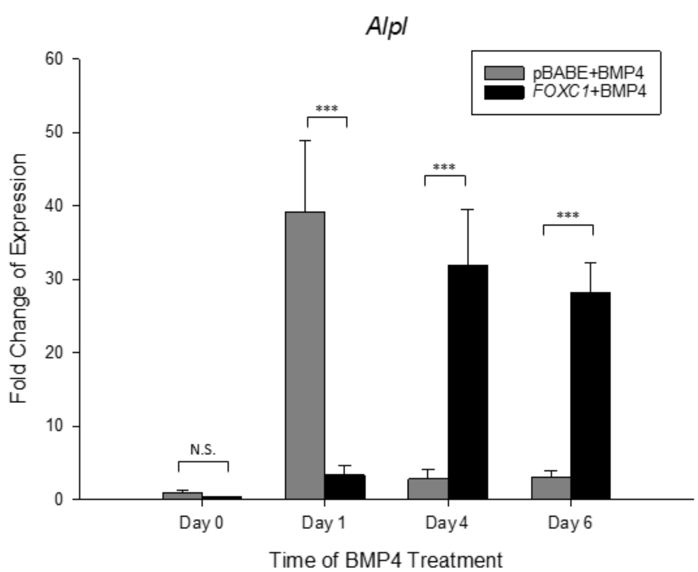
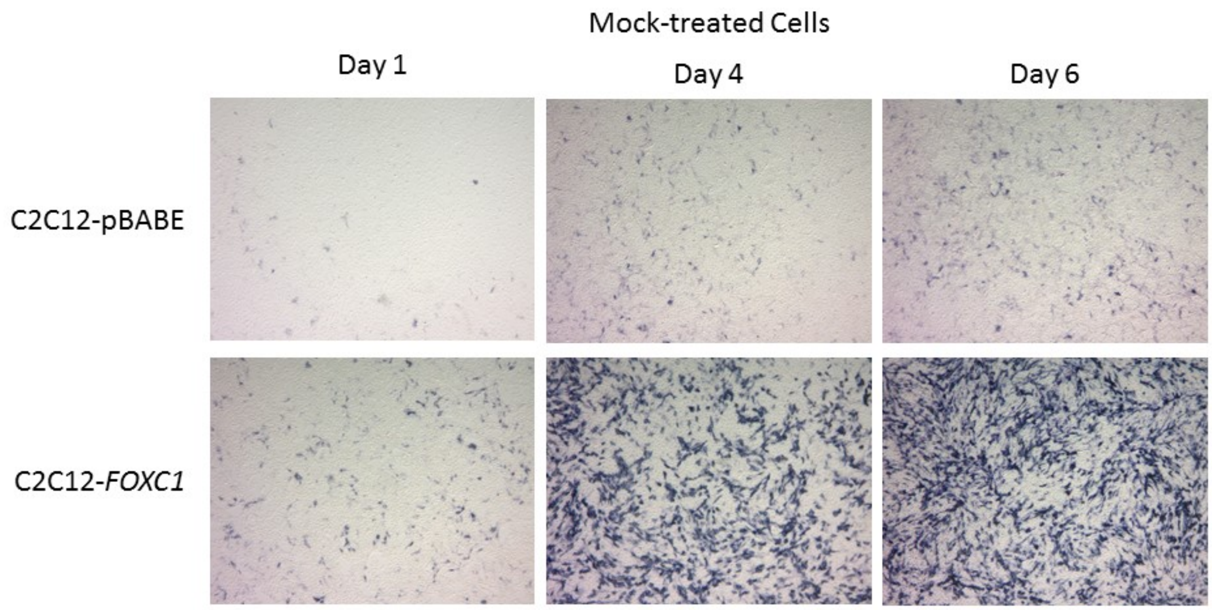


Figure 3.15

FOXC1 overexpression increases Alpl production in C2C12 myoblasts. **A.** C2C12-pBABE or C2C12-pBABE-*FOXC1* cells were stained after mock treatment for 1, 4, and 6 days. Blue in images represent Alpl protein, with increased blue corresponding to more Alpl. **B.** Quantification of Alpl staining using percent area stained. Asterisks represent statistical significance. **: P < 0.01; ***: P < 0.001.

Figure 3.15

A.



B.

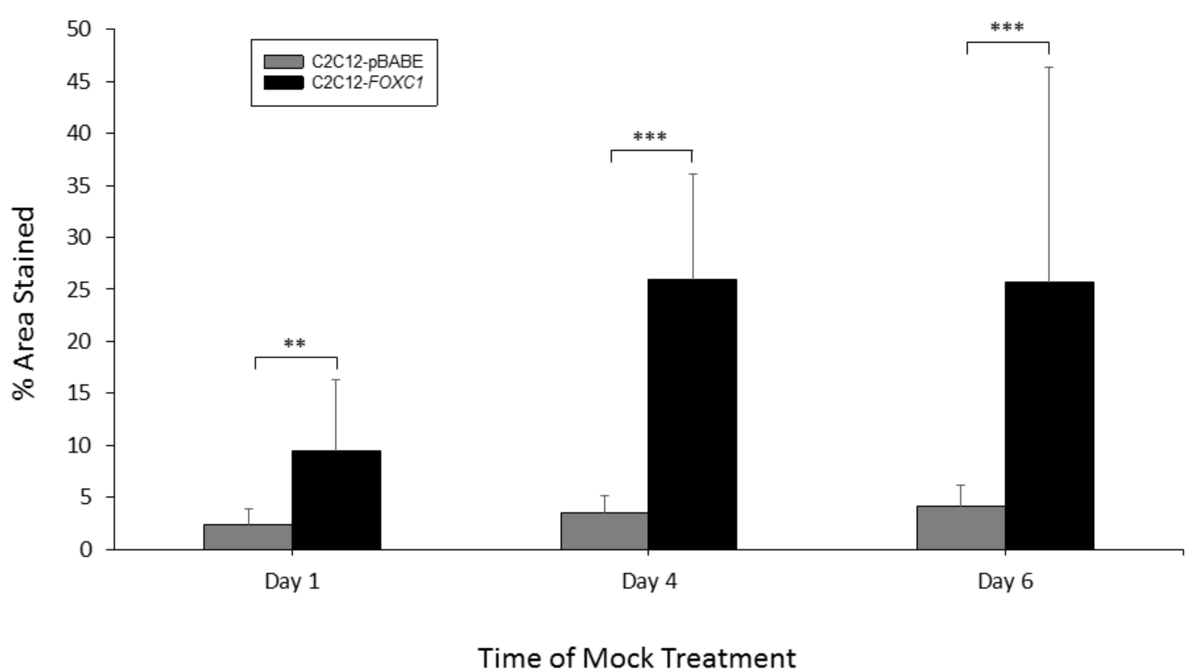
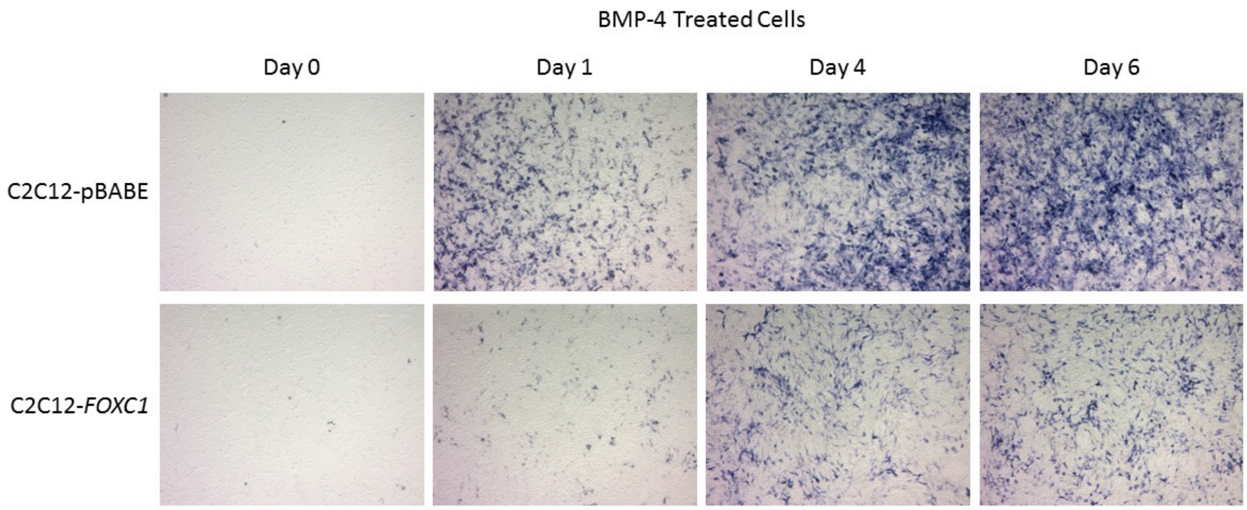


Figure 3.16

FOXC1 overexpression reduces BMP-4 induced Alpl production during osteoblast differentiation. **A.** C2C12-pBABE or C2C12-pBABE-*FOXC1* cells were stained at the time of BMP-4 treatment, and at 1, 4, and 6 days of treatment. Blue in images represent Alpl protein, with increased blue corresponding to more Alpl. **B.** Quantification of Alpl staining using percent area stained. Asterisks represent statistical significance. **: $P < 0.01$; N.S.: not significant.

Figure 3.16

A.



B.

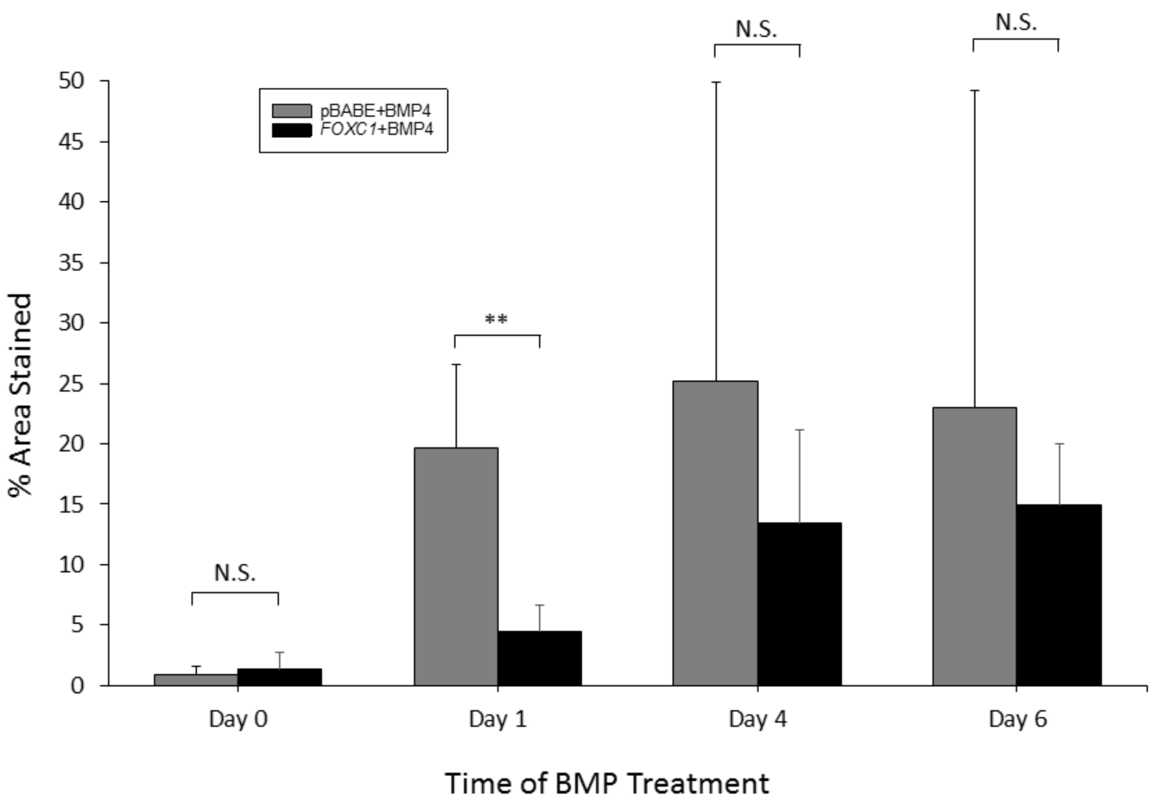
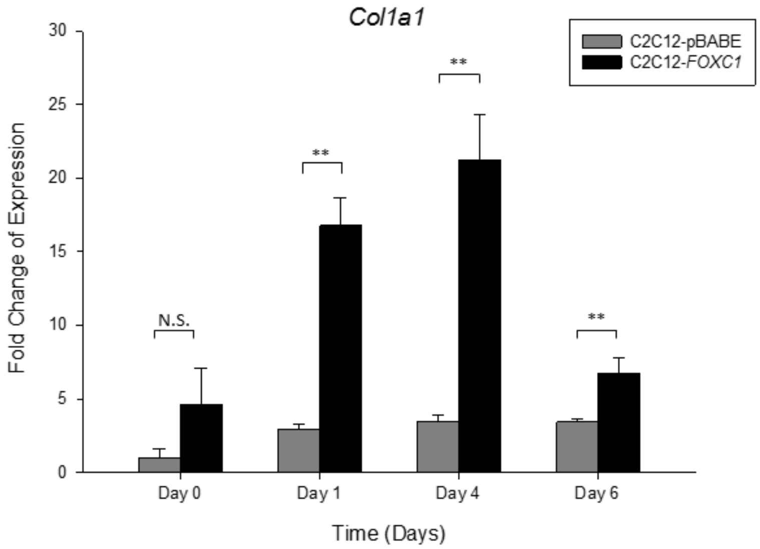


Figure 3.17

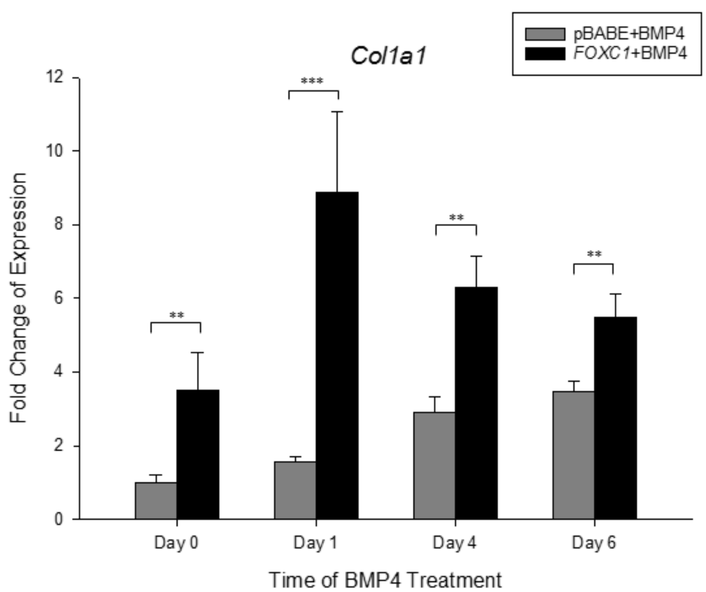
FOXC1 overexpression increases *Col1a1* expression during osteoblast differentiation. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.17

A.



B.



show a gradual incline in expression (Fig 3.18A). Conversely, there is no change in *Opn* expression between the two cell types under BMP induction until day 6, when *Opn* is increased in *FOXC1* overexpressing cells (Fig 3.18B). Finally, there is a reduction in *Osx* expression at day 6 in mock-treated C2C12-pBABE-*FOXC1* cells relative to control cells (Fig 3.19A). Levels remained unchanged in both cells until then. When treated with BMP there is no change in *Osx* expression between the two cells (Fig 3.19B).

3.5.3 *C2C12 passage number impact in response to ectopic* FOXC1 *expression*

As biological replicates were completed in the time course assay, inconsistencies in gene expression was observed depending on how long the cells were cultured. As passage age progressed, cellular responses to *FOXC1* overexpression and BMP treatment changed. Figures 3.6-3.19 are results from C2C12 cells of lower passage number: 8-14. Figures 3.20-3.21 are time course qPCR results from C2C12 cells of higher passage: 15-22. In older passage C2C12-pBABE cells some genes such as *Id1*, *Runx2*, and *Dlx3* are not induced by BMP-4 whereas *Hey1* induction is delayed (Figure 3.20 compare 3.11B, 12B, 13B, 14B). Older passage *FOXC1* overexpressing cells show delayed induction of *Id1* and *Dlx3*, whereas *Runx2* is induced at day 4 and *Hey1* is not induced by BMP-4 treatment. Osteoblast markers are also affected in older passage cells (Figure 3.21 compare 3.15B, 3.17B, 3.18B, 3.19B). In pBABE control cells *Alpl* is slightly upregulated at day 1 of BMP treatment, whereas in C2C12-pBABE-*FOXC1* cells *Alpl* is reduced relative to younger passage cells, though response to BMP occurs earlier in the time course (Figs 3.15B and 3.21). There is an increase in *Opn* expression at day 4 compared to young passage cells in C2C12-pBABE cells (Figs 3.18B and 3.21). In C2C12-pBABE-*FOXC1* cells *Opn* has no change in expression until day 6 when a slight increase in expression is observed. There is no change from young to older passage *Col1a1* response to BMP-4 treatment (Figs

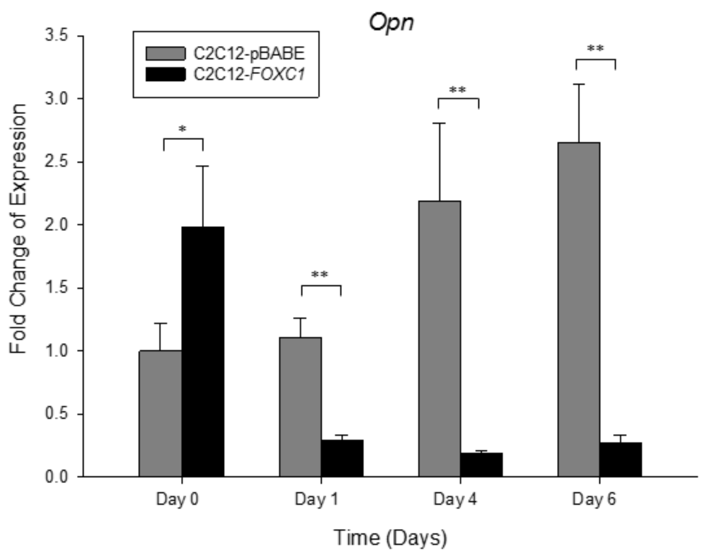
3.17B and 3.21). In *FOXC1* overexpressing cells *Col1a1* is activated by BMP-4 to lower levels than in younger cells (Figs 3.17B and 3.21). *Osx* is not detected above background in older cells preventing any comparisons (Fig 3.21).

Figure 3.18

FOXC1 overexpression changes *Opn* expression in untreated and BMP-4 treated C2C12 cells. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.18

A.



B.

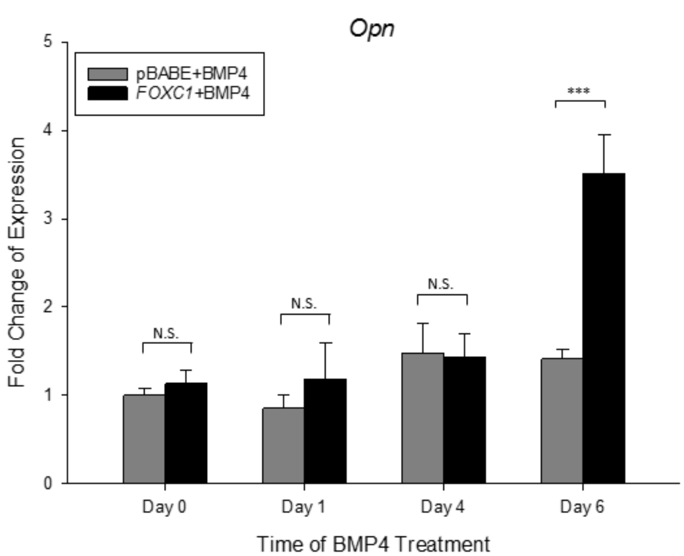
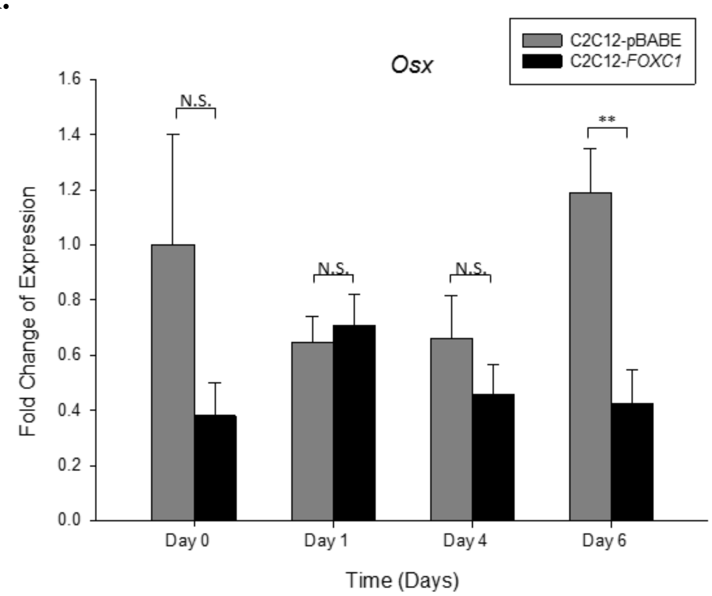


Figure 3.19

FOXC1 overexpression reduces *Osx* expression late in ectopic osteoblast differentiation. **A.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after mock treatment for 0, 1, 4, and 6 days. **B.** Transcripts amplified from C2C12-pBABE and C2C12-pBABE-*FOXC1* cells after BMP-4 treatment for 0, 1, 4, and 6 days. Significance was determined by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant.

Figure 3.19

A.



B.

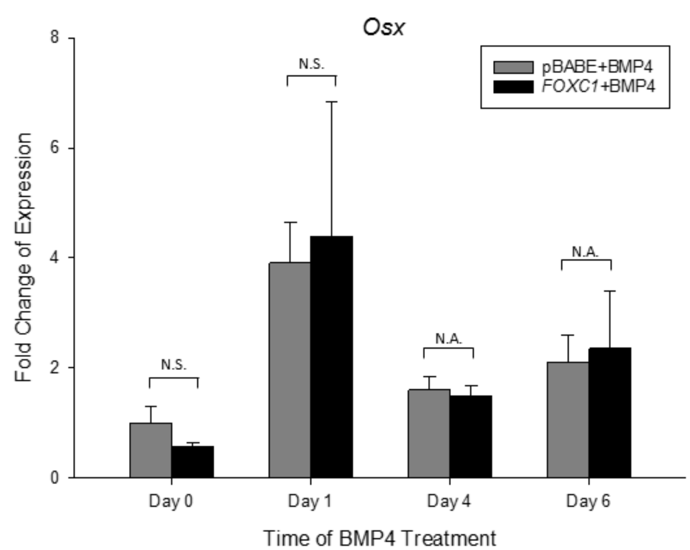


Figure 3.20

The impact of *FOXC1* overexpression on BMP-SMAD gene expression is changed in older passage cells. cDNA from cells of passage number 15-22 was amplified using primers for *Id1* **(A)**, *Runx2* **(B)**, *Dlx3* **(C)**, and *Hey1* **(D)**. Data was analyzed by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant; N.A.: not applicable.

Figure 3.20

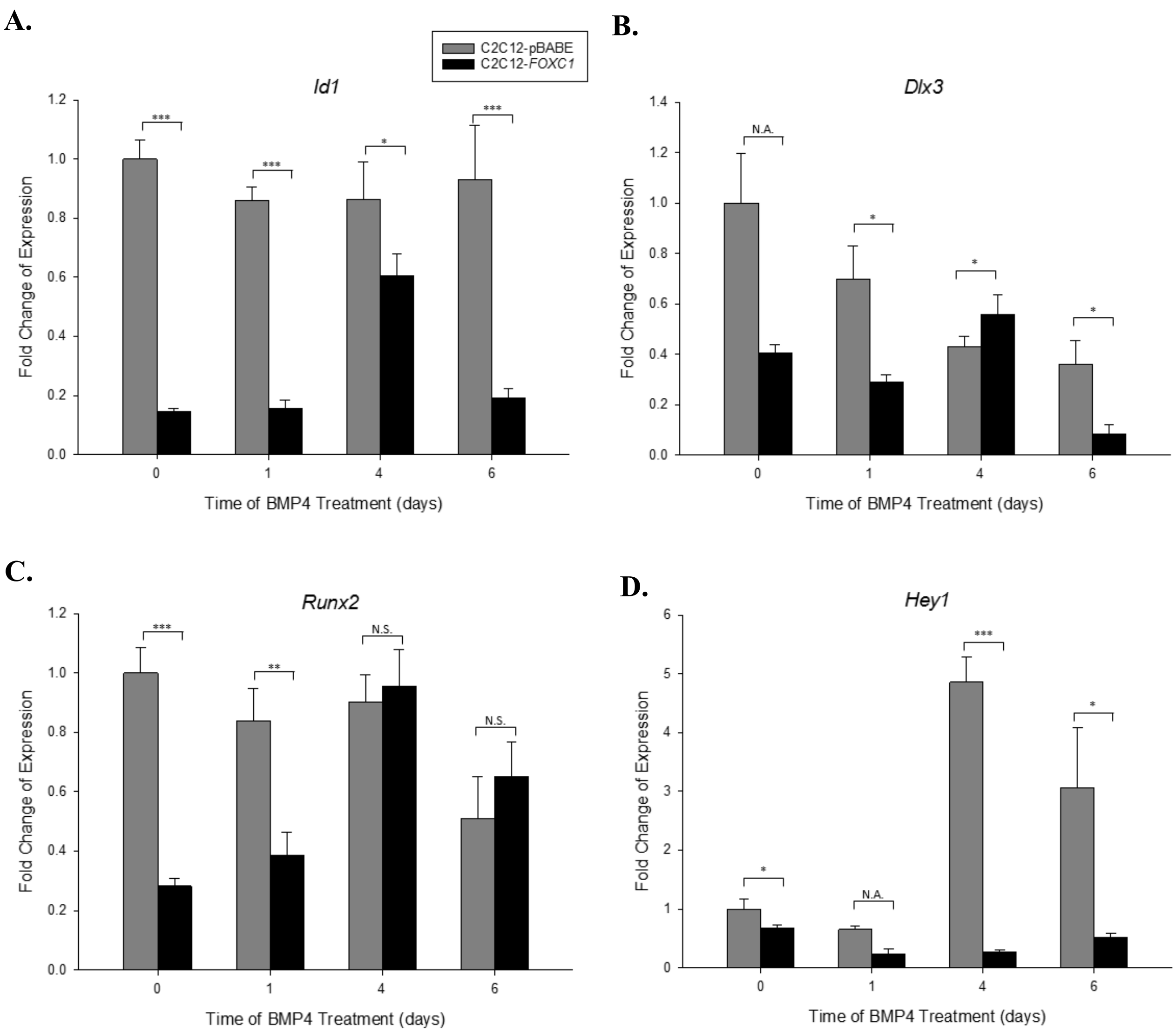
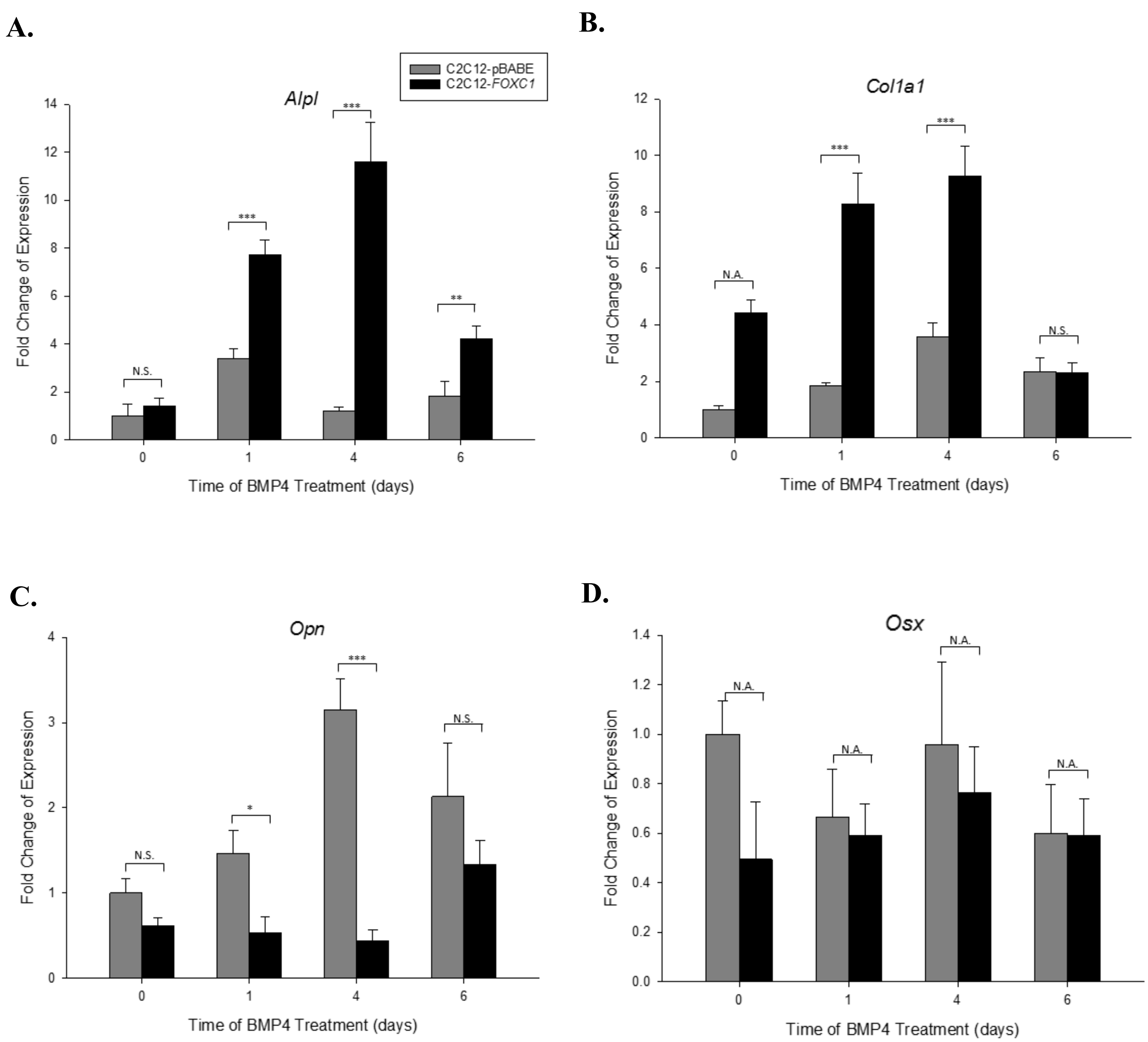

A.
C2C12-pBABE
C2C12-FOXC1
Id1
Fold Change of Expression
Time of BMP4 Treatment (days)
***
*
N.A.
B.
Dlx3
C.
Runx2
**
N.S.
D.
Hey1

Figure 3.21

The impact of *FOXC1* overexpression on osteoblast marker expression in older passage cells. Cells of passage number 15-22 were cultured in 6-well plates for 48 hours. The cells were then washed in 1x PBS and treated with 50 ng/mL BMP-4 or mock treatment for 0, 1, 4, or 6 days. RNA was then extracted from the cells and qRT-PCR carried out. Specific gene products were amplified using primers for *Alpl* **(A)**, *Col1a1* **(B)**, *Opn* **(C)**, and *Osx* **(D)**. Data was analyzed by CFX Manager software. Asterisks represent statistical significance. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; N.S.: not significant; N.A.: not applicable.

Figure 3.21


A.
C2C12-pBABE
C2C12-*FOXC1*
Alpl
Fold Change of Expression
Time of BMP4 Treatment (days)
N.S.
***
***
**
B.
Col1a1
Fold Change of Expression
Time of BMP4 Treatment (days)
N.A.
***
***
N.S.
C.
Opn
Fold Change of Expression
Time of BMP4 Treatment (days)
N.S.
*
***
N.S.
D.
Osx
Fold Change of Expression
Time of BMP4 Treatment (days)
N.A.
N.A.
N.A.
N.A.

Chapter Four: Discussion

4.1 Summary of the Findings

FOX proteins interact with TGF-β and BMP signalling networks to impact development, cell differentiation as well as proliferation (Astorga & Carlsson, 2007; Blount et al., 2009; Fujita et al., 2006; Grainger et al., 2010). TGF-β and BMP signalling overlap with FOXC1 expression in many aspects of biology including bone formation (Hopkins et al., 2015; Kume et al., 1998; Kume et al., 2001; Mirzayans et al., 2012; Rice et al., 2003). Therefore, this work hypothesized that FOXC1 interacts in BMP-SMAD signalling to affect bone formation.

The present work tested the impact of FOXC1 on BRE activation under BMP-4 and SMAD induction. FOXC1 generally activates its transcriptional targets and is important to proper bone formation (Kume et al., 1998); therefore, FOXC1 was expected to enhance BMP-SMAD signalling and therefore activation of *Id1* BRE. FOXC1 expression reduces BRE activation by both BMP-4 and SMAD treatment (Figs 3.1, 3.2). SMAD induction of BRE jumpstarts BMP-SMAD signalling without the need for BMP-4, BMP receptors or other factors. Because FOXC1 inhibits SMAD-induced BRE activation, the possible ways FOXC1 interacts in the pathway can be narrowed down to protein-protein interactions within the nucleus or DNA-protein interactions. The importance of individual FOXC1 domains to BRE inhibition was then tested. Using FOXC1 constructs missing functional domains and an S131L FOXC1 mutant, this work shows that the N-AD, IHD, and FHD are important to reduce BRE activation (Figs 3.3A, 3.4A). To test if FOXC1 affects BMP-SMAD signalling endogenously, C2C12-pBABE-*FOXC1* cells were treated with BMP-4 and changes in *Id1* mRNA were observed. QRT-PCR shows that FOXC1 inhibits endogenous *Id1* expression in the presence and absence of BMP-4 (Fig 3.6). To determine if FOXC1 affects BMP-SMAD signalling globally, other BMP-SMAD targets were observed. Also, since activated BMP-SMAD signalling induces osteoblast differentiation in

C2C12 cells, changes in osteoblast marker gene expression were evaluated. *Runx2* expression increased in *FOXC1* overexpressing cells when treated with BMP-4; however, BMP-4 treatment did not result in an increase in *Runx2* expression in wildtype cells (Fig 3.7A). *Alpl* and *Col1a1* expression increased regardless of BMP treatment in *FOXC1* overexpressing cells (Fig 3.8A, B).

Finally, the present work longitudinally observed myoblasts with and without BMP-4 treatment to determine the impact FOXC1 has on osteoblast differentiation. When treated with BMP-4, *Id1* expression was consistently reduced whereas *Runx2* expression was only reduced immediately after BMP-4 treatment in C2C12-pBABE-*FOXC1* cells relative to control (Fig 3.11 B, 3.12B). Conversely, *Dlx3* expression increased at day 4 of the time course (Fig 3.13B). When C2C12-pBABE-*FOXC1* cells were not treated with BMP-4, a consistent reduction in *Id1* expression was observed (Fig 3.10A), whereas *Dlx3* expression was increased early in the time course (Fig 3.12A) compared to control cells, suggesting FOXC1 may cause ectopic changes in BMP-SMAD signalling.

Alpl expression was increased later in C2C12-pBABE-*FOXC1* cells compared to control cells treated with BMP-4, however, the increase in expression is also sustained later into the time course in C2C12-pBABE-*FOXC1* cells (Fig 3.14B). In untreated C2C12-pBABE-*FOXC1* cells, *Alpl* expression is increased consistently in the time course (Fig 3.14A). Changes in Alpl staining is also observed during the time course (Fig 3.15, 3.16). Control cells treated with BMP-4 stain for Alpl more robustly than C2C12-pBABE-*FOXC1* cells on (Fig 3.16). However, *FOXC1* overexpression results in an increase in Alpl production without BMP-4 compared to control cells (Fig 3.15).

In BMP-4 treated C2C12-pBABE-*FOXC1* cells, *Col1a1* expression is consistently higher than control cells (Fig 3.17B), whereas no change is observed in *Osx* expression (Fig 3.19B), and *Opn* expression is increased late in the time course (Fig 3.18B) compared to control cells. *Col1a1* expression is also higher in untreated C2C12-pBABE-*FOXC1* cells compared to control cells (Fig 3.17A). However, *Opn* expression is initially increased, but later decreased in *FOXC1* overexpressing cells (Fig 3.18A).

4.2 Interpretation of Findings

4.2.1 FOXC1 *inhibits* BMP-*induced BRE activation*

When FOXC1 production is increased in U2OS cells BRE activation is inhibited both with and without BMP-4 treatment (Fig 3.1). Because *FOXC1* expression increased in BMP-treated C2C12 cells in the literature (Hopkins et al., 2015), this finding is surprising as it was expected that FOXC1 would activate BMP-SMAD signalling in C2C12 myoblasts. Also, as hypothesized by Hopkins et al. (2015) this may suggest that *FOXC1* expression is temporally sensitive, and may promote the initial dedifferentiation of partially committed mesenchymal progenitor cells before the commitment to osteoblast. In this case, FOXC1 may serve to temper BMP-induced BRE activation and reduce the U2OS proliferation rate determined in part by *Id1*.

FOXC1 and FOXC2 share close FHD and IHD homology and some functions and targets overlap during development (Fujita et al., 2006; Kume et al., 2000; Kume et al., 2001; Sasman et al., 2012; Seo & Kume, 2006; Seo et al., 2006; Wilm et al., 2004). The two proteins also may be targeted by similar proteins for proteomic regulation (Danciu et al., 2012). FOXC2's impact on BMP-induced BRE was also evaluated as a way of determining if there is redundancy between the two proteins in *Id1* BRE response. BRE activation with and without BMP-4 is not different between FOXC2- and FOXC1-expressing U2OS cells. Therefore, it is hypothesized that these proteins may have a common function in BMP signalling. FOXC1 and FOXC2 have homology in the IHD and FHD (Saleem et al., 2004). Thus, it may be hypothesized that a homologous domain between the two proteins contributes to the similar inhibitory activity on the BRE.

There are a few mechanisms by which FOXC1 may inhibit BMP activation of BRE. It may bind on or near the SMAD binding site of the BRE and sterically prevent SMAD complex localization. The *Id1* BRE used in these experiments does not have a FOXC1 binding site; however, FOXC1 may bind the plasmid vector and sterically hinder SMADs from binding the BRE. The proximal promoter region of *Id1* does contain a FOXC1 binding site upstream of the transcription start site, and downstream from the BRE (Fig 4.1). If the inhibitory activity of FOXC1 is occurring in the promoter region, FOXC1 may bind its target site, recruit the BRE-bound SMAD complex through DNA looping, and recruit a repressor protein to switch the common transcriptional activation activity of FOXC1 to an inhibitory one (Fig 4.1A). Alternatively, FOXC1 may bind the SMAD complex and sequester it from the BRE perhaps to a new target or competitively inhibit SMAD complex formation by binding R- or Co-SMAD at its protein binding site (Fig 4.1B). Finally, FOXC1 binding to R- or Co-SMAD may change the function of the SMAD complex from activating transcription to inhibiting it.

4.2.2 Functional domains and location of **FOXC1** *inhibitory activity*

FOXC1 and FOXC2 production both result in reduced levels of BRE activation in U2OS cells, suggesting some functional redundancy within the two proteins is responsible for this activity (Fig 3.1). Also, FOXC1 inhibition of the BRE is a result of either protein-protein or protein-DNA interactions as indicated by figure 3.2. Therefore, it was hypothesized that region(s) within the protein play an important role in this inhibitory activity. In Figure 3.3A when the N-AD is deleted from FOXC1, both basal and BMP-induced BRE activity returns to control levels. This suggests that the N-AD is crucial for wildtype FOXC1 activity with regards to BMP-dependent BRE activation. When the C-terminal end including the IHD of FOXC1 is deleted, basal levels of BRE are significantly increased, suggesting the IHD inhibits BRE activation. However, BMP-4 induction does not induce BRE further in FOXC1 1-215 (Fig 3.3A), which also supports the suggestion that N-AD is required for inhibition of BMP-induced BRE activation. Finally, when only the FHD is present in the cells, basal levels of BRE are increased and BMP induction induces BRE levels further. This suggests that the FHD is not sufficient to inhibit BRE activation and thus FOXC1 inhibition of transcription is not likely due solely to FOXC1 binding the BRE and sterically hindering SMAD complex binding. It is possible that full length FOXC1 sterically hinders SMAD binding to their targets. However, it is unlikely; as N-AD deletion – a comparably short sequence of amino acids – returns FOXC1 function to control levels. Therefore, an intact IHD is essential for general, BMP-independent inhibition of BRE.

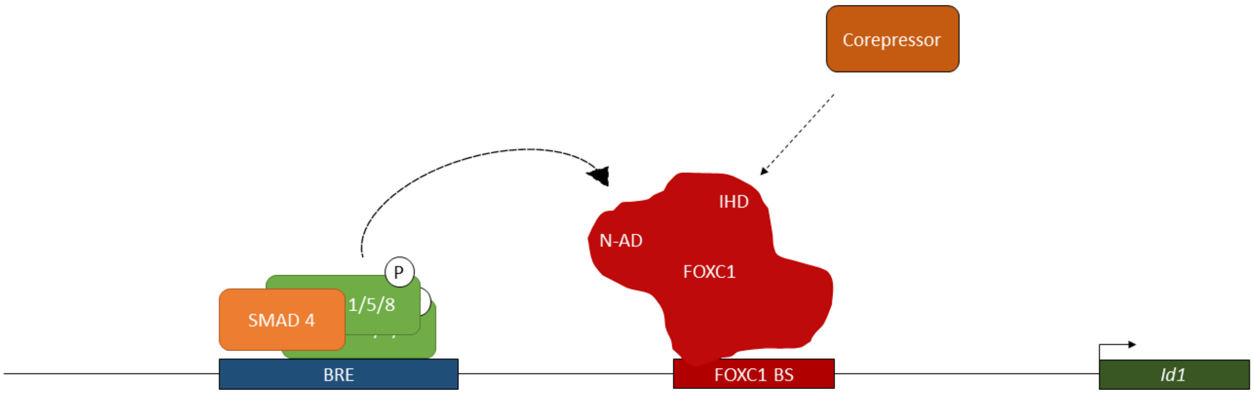
A point mutation in the FHD of FOXC1, S131L, was previously developed and analyzed for changes in protein function (Saleem et al., 2001). It was found that FOXC1 S131L protein could still enter the nucleus, though its DNA binding capability was greatly reduced. FOXC1 S131L was included in a luciferase transactivation assay under BMP induction to determine if DNA binding ability was necessary for FOXC1-mediated inhibition of BRE activation (Fig 3.4A, B). FOXC1 S131L is not able to inhibit BRE activation in U2OS cells (Fig 3.4A) but is significantly different from neither control nor wildtype BRE activation (Fig 3.4B).

Figure 4.1

Possible models of FOXC1 activity affecting BRE activation. FOXC1 may interact at the DNA or protein level to affect *Id1* gene expression through BRE activation. **A.** A model of one possibility of FOXC1 inhibition of *Id1* BRE if FOXC1 interacts at the DNA level. **B.** If FOXC1 is inhibiting BRE activation at the protein level, it may be interacting with a repressor or SMAD complex protein through active or passive inhibition.

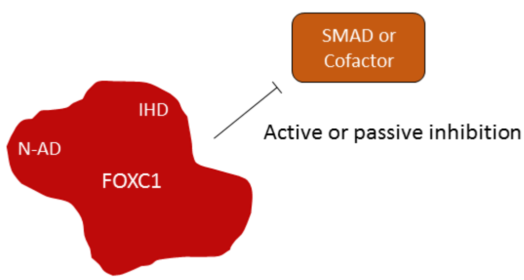
Figure 4.1

A.



Corepressor
IHD
N-AD
FOXC1
P
1/5/8
SMAD 4
BRE
FOXC1 BS
Id1

B.



SMAD or
Cofactor
IHD
N-AD
FOXC1
Active or passive inhibition

Transactivation assays are not specific cell to cell, but are an average of the cell population's response to transfection, and includes the cells that take in 0, 1, 2, or all 3 plasmids. C2C12 cells are not transfected as easily as U2OS cells, and these results may be an artifact of few cells having all plasmids necessary for accurate measurements.

To determine how FOXC1 may inhibit *Id1* BRE activation, SMAD-4 and SMAD-5 were co-transfected into U2OS cells (Fig 3.2). FOXC1 introduction to the SMAD-activated BRE-luciferase reduces BRE-activation levels to control levels. Because SMAD induction of BRE is a model of BMP-SMAD signalling downstream of SMAD activation, FOXC1 inhibition occurs through interaction with other transcription factors like SMAD-4 or SMAD-5; or by interaction at the DNA level. This is in support of others systems wherein FOXC1 binds proteins or DNA to affect change in gene expression (Berry et al., 2006; Berry et al., 2008; Han et al., 2015; Mirzayans et al., 2012; Xia et al., 2013). FOXC1 binds DNA and some proteins via the FHD, further supporting the evidence in Figures 3.1 and 3.3 that the FHD may be important for inhibition of BRE (Saleem et al., 2004).

4.2.3 **FOXC1** ***inhibits Id1 expression***

FOXC1 inhibits *in vitro Id1* BRE activation, thus I hypothesized that endogenous *Id1* mRNA levels will be reduced in cells overexpressing *FOXC1*. Using C2C12-pBABE-*FOXC1* cells allowed for the stable expression of *FOXC1* in mouse myoblast cells capable of osteoblast differentiation under BMP-4 treatment. Both un-induced and BMP-induced C2C12-pBABE-*FOXC1* cells show consistently decreased *Id1* mRNA levels compared to both wildtype and lentiviral control C2C12 cells (Fig 3.6, 3.10, 4.2, 4.4,). This is in agreement with the transactivation assay findings. This shows that the same response to *FOXC1* overexpression occurs in C2C12 cells and the BRE-luciferase assays. The general inhibition of *Id1* by FOXC1 may be momentarily overcome by BMP-SMAD signalling, as no difference between the two cells is observed on days 1 and 4 in figure 3.10B. FOXC1 may be inhibiting BMP-SMAD signalling globally or just at *Id1*. However, this experiment does not give more information regarding the nature of FOXC1's interaction in the signalling pathway.

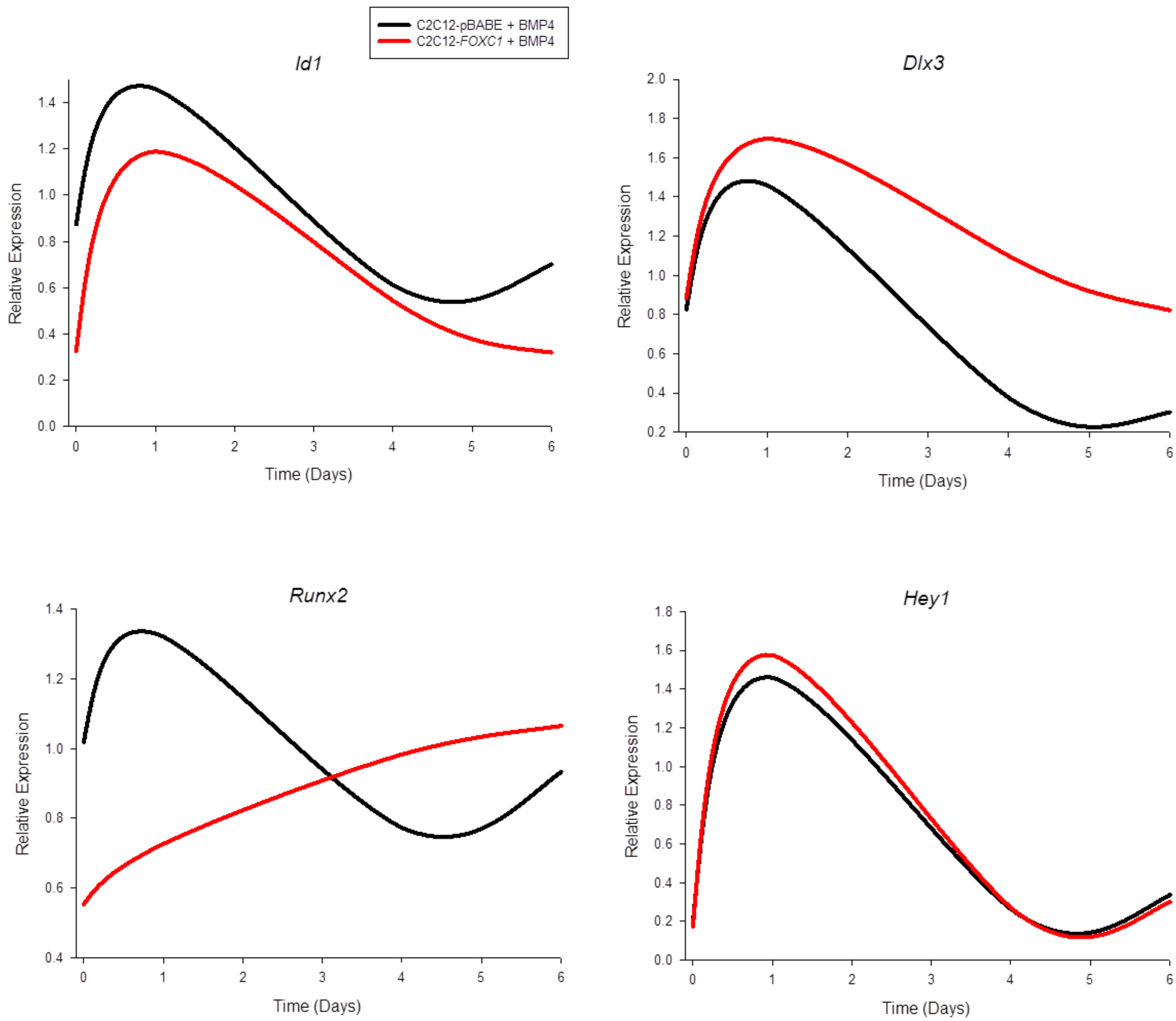
4.2.4 **FOXC1** ***overexpression on*** **BMP-*induced osteogenesis***

To understand FOXC1's impact on BMP-SMAD signalling in myoblasts undergoing osteoblast differentiation, genes upregulated by BMP-SMAD signalling in the literature were investigated (Figs 3.7, 3.11, 3.12,3.13). Summary graphs are presented in Figure 4.2. *Runx2* expression is not induced by BMP-4 in control cells in the present work (Figs 3.7A, 3.11B). However, *Runx2* is initially inhibited when *FOXC1* is overexpressed (Fig 3.11B). Using one FOXC1 binding sequence 5'-GTAAATAAA-3' to search in the proximal regulatory region of *Runx2*, three prospective FOXC1 binding sites were located within 10 kb upstream of *Runx2* transcriptional start site. Four targets total were found in the proximal regulatory region using another FOXC1 target sequence 5'-GTAAATA-3'. Therefore, FOXC1 may initially prevent *Runx2* activation by BMP-SMAD signalling through steric hindrance by binding *Runx2*'s proximal regulatory region. However, considering *Id1* is continually inhibited by FOXC1 and *Runx2* is only inhibited when cells are treated by BMP-4, the mechanism for inhibiting the two genes likely differs. For example, FOXC1 may only bind active pSMADs at the *Runx2* proximal regulatory region and reduce gene expression, whereas FOXC1 inhibits *Id1* irrespective of BMP-SMAD signalling.

Figure 4.2

FOXC1 deregulates BMP-SMAD pathway gene expression during BMP-4 induced osteoblast differentiation. Summary line graphs of results provided by Figures 3.10B, 3.11B, 3.12B, and 3.13B. Expression values are relative to expression of three control genes: *Hprt*, *Actin B*, and *Gapdh* at day 0 of BMP-4 treatment in C2C12-pBABE cells.

Figure 4.2

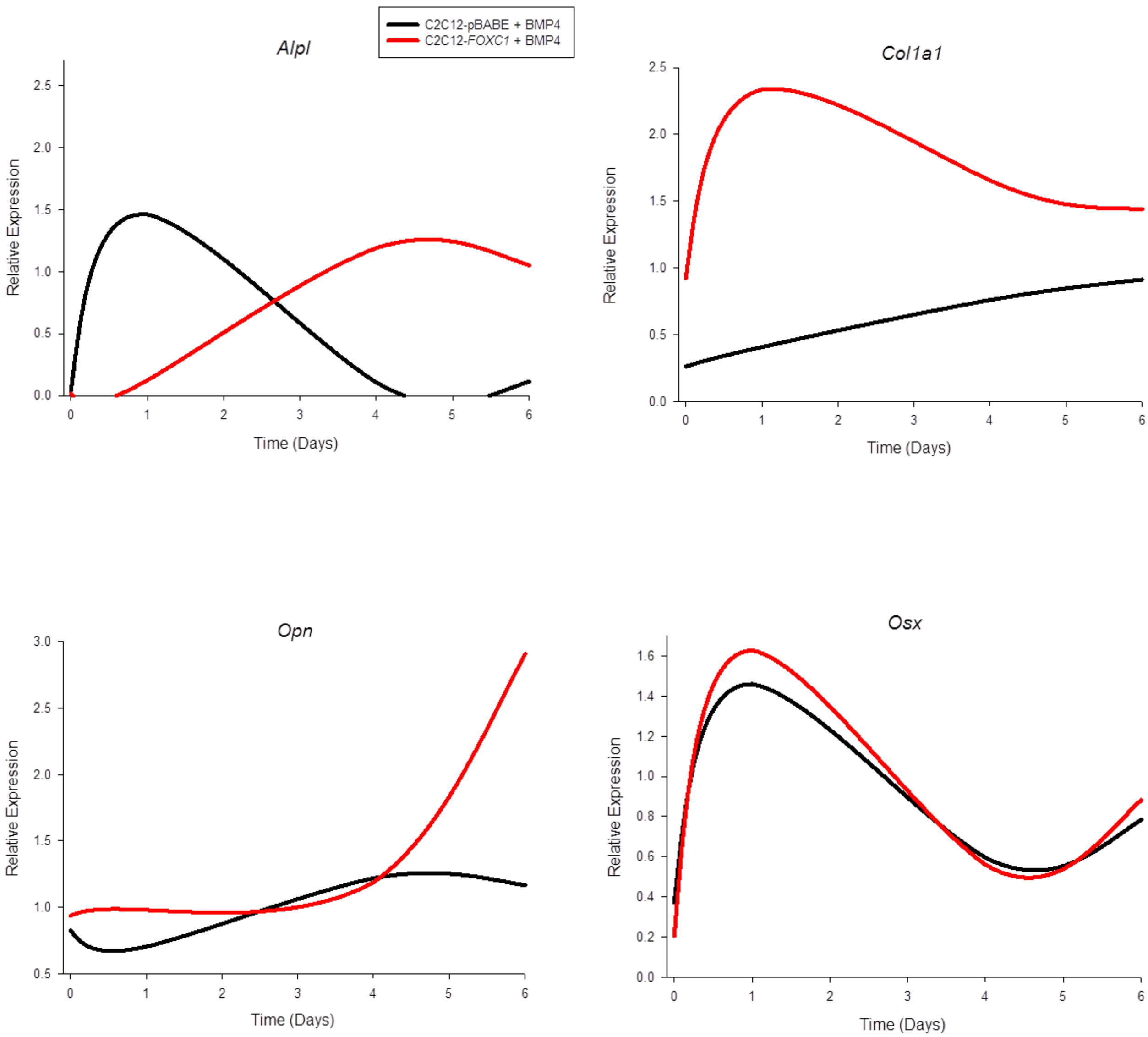


C2C12-pBABE + BMP4
C2C12-FOXC1 + BMP4
Id1
Dlx3
Runx2
Hey1
Relative Expression
Time (Days)

Figure 4.3

FOXC1 overexpression deregulates osteoblast marker expression during BMP-4 induced osteoblast differentiation. Summary line graphs of results provided by Figures 3.14B, 3.17B, 3.18B, and 3.19B. Expression values are relative to expression of three control genes: *Hprt*, *Actin B*, and *Gapdh* at day 0 of BMP-4 treatment in C2C12-pBABE cells.

Figure 4.3


C2C12-pBABE + BMP4
C2C12-*FOXC1* + BMP4
Alpl
Col1a1
Opn
Osx
Relative Expression
Time (Days)

Dlx3 expression is increased in C2C12-pBABE-*FOXC1* cells treated with BMP-4 compared to controls (Figs 3.12B, 4.2) whereas no change in *Hey1* expression is observed (Figs 3.13B, 4.2). This information, together with the initial inhibition of *Runx2* and sustained inhibition of *Id1* shows that FOXC1's impact on the BMP-SMAD signalling network is not unilateral, but varies greatly with the target. Thus a deregulation of BMP-SMAD signalling is observed when *FOXC1* expression is not reduced in the cell.

To determine if FOXC1 impacts BMP-induced osteoblast differentiation *Alpl*, *Col1a1*, *Opn* and *Osx* were analyzed (Figs 3.14B, 3.17B, 3.18B, 3.19B). Summary graphs are presented in Figure 4.3. *Alpl* expression appears delayed and prolonged in C2C12-pBABE-*FOXC1* cells relative to control cells, which alone may suggest osteoblast differentiation is extended temporally when *FOXC1* is ectopically expressed. However, when *Col1a1*, *Opn*, and *Osx* expression are considered together, a dysregulation of BMP-induced osteoblast differentiation appears to be occurring (Fig 4.3).

The initial spike in *Alpl* expression in C2C12 cells treated with BMP-4 is higher and the corresponding decrease thereafter more extreme than expected (Figs 3.14B), though it can be explained by the time-jump snapshot nature of experiment. The parabolic curve may still exist, and the samples from day 1 and day 4 may simply be the peak and valley of the normal expression (Fig 3.14B, 4.3). If that is the case, the lack of strong Alpl staining at day 1 of BMP-4 treatment in C2C12-pBABE-*FOXC1* cells (Fig 3.16) is a result of not all the mRNA present in the qPCR being translated into protein for Alpl staining. The interesting trend of lower Alpl staining as BMP-4 treatment progresses from day 1 to day 6 relative to untreated C2C12-pBABE-*FOXC1* cells may be the result of BMP-SMAD signalling negatively impacting FOXC1-mediated osteoblast differentiation (Fig 3.15, 3.16). A product of BMP-SMAD signalling may inhibit FOXC1 function when levels are high, resulting in slower osteoblast differentiation in C2C12-pBABE-*FOXC1* cells. Alternatively, FOXC1 may be sequestered away from its inhibitory activity when BMP-SMAD signalling it present. Filamin-A (FLNA) is capable of inhibiting FOXC1 transcriptional activity by binding and sequestering FOXC1 to heterochromatin (Berry et al., 2005). This or a similar effect may occur when BMP signalling is induced to control excessive FOXC1 activity and ensure proper bone patterning during development.

Interestingly, *Col1a1* expression increased as a result of *FOXC1* overexpression in cells treated with BMP-4 (Figs 3.8B, 3.17B, 4.3). Under BMP induction, *Col1a1* is expressed when Runx2, Dlx3 or Dlx5 bind its promoter (Ducy et al., 1997, Hassan et al., 2004, Tadic et al., 2001). However, *Col1a1* expression is increased despite a decrease in *Runx2* expression (Figs 3.11B, 3.17B). Finally, *Osx* expression is unchanged in *FOXC1*-overexpressing cells treated with BMP-4 relative to controls (Figs 3.8C, 3.19B). Because *Osx* is a downstream target of BMP-SMAD signalling (Lee et al., 2003), the decrease in *Runx2* expression (Fig 3.11B, 4.3) should have resulted in a decrease in *Osx* expression when *FOXC1* is overexpressed. The lack of change in *Osx* expression suggests that BMP-SMAD signalling pathway is not proceeding as normal from *Runx2* onward. These findings suggest that FOXC1 is also affecting osteoblast differentiation independent of BMP-SMAD signalling which will be discussed in the next section.

Cellular response to BMP-4 was also subject to how long the cells were passaged. The difference noticed between older and younger passage cells and their response to BMP-4 induction and *FOXC1* overexpression (Figs 3.20 and 3.21) suggests that C2C12 cells, whether stably expressing *FOXC1* or not, should be observed carefully for changes in morphology, media condition, and rate of proliferation after 7 weeks of culturing. C2C12, C2C12-pBABE, and C2C12-pBABE-*FOXC1* cells were cultured for an unknown period of time before storage in liquid nitrogen. Therefore, the passage numbers of 8-14 for young cells and 15-22 for old cells is somewhat arbitrary, and the cells used in the time course assay were closely monitored after changes in gene expression and Alpl staining were noticed between old and young C2C12 cells.

4.2.5 **FOXC1** ***overexpression on ectopic osteogenic differentiation***

Interestingly, the results of *FOXC1* overexpression in untreated cells show that FOXC1 is capable of affecting the expression of BMP-SMAD signalling and osteoblast marker genes independent of BMP-4 treatment (Figs. 3.10A-3.19A). Summary graphs are presented in Figures 4.3 and 4.4. A summary of gene expression in BMP- and mock-treated cells is presented in Table 4.1. *Id1* is consistently inhibited, whereas *Runx2* expression does not seem to be affected when *FOXC1* is overexpressed (Figs 3.10A, 3.11A, 4.4). *FOXC1* overexpression increases *Dlx3* expression early in serum-starved myoblasts, which decreases to levels seen in control cells (Figs 3.12A, 4.4). Similarly, *Hey1* expression in serum starved C2C12 cells differs from C2C12-pBABE-*FOXC1* overexpressing cells (Figs 3.13A, 4.4), C2C12-pBABE-*FOXC1* cells show a similar trend in *Hey1* expression to *Dlx3* expression, though the levels of expression differ.

The change from a smooth sigmoidal curve in *Hey1* expression in control cells to a plateaued descent in C2C12-pBABE-*FOXC1* cells further illustrates the broad changes that may be happening in the cells when *FOXC1* expression is not turned down (Fig 4.4). Temporal coordination of Notch signalling with BMP- or TGF-β signalling in these cells may be de-regulated with consistently increased FOXC1 production, which may result in Notch and BMP signalling gene *Hey1* expression increasing immediately after serum starvation and decreasing shortly thereafter in C2C12-pBABE-*FOXC1* cells (Fig 4.4). However, changes to *Dlx3* and *Hey1* expression may be explained by downstream effects unrelated to BMP signalling (Figs 3.12, 3.13). Dlx3, in addition to being a crucial component of osteogenesis, is also important to hair follicle maturation and self-renewal (Hwang et al., 2008). FOXC1's important role in hair stem cell regulation has also been described recently (Wang et al., 2016b). Thus the temporary increases observed in C2C12-pBABE-*FOXC1* cells may be an artifact of their cooperation in other pathways related to cell regeneration (Fig 3.12B).

The increase in *Alpl* expression without BMP-4 treatment for 24 hours (Fig 3.8A) is of great interest because *Alpl* is not part of the BMP-SMAD pathway and an increase in expression can be explained by FOXC1 inducing osteoblast differentiation itself, rather than through BMP-dependent SMAD signalling. *Alpl* expression as a response to increased *FOXC1* expression corresponds to increased Alpl staining in untreated cells (Fig 3.14, 4.5). Thus, C2C12 cells stably expressing *FOXC1* are indeed differentiating into pre-osteoblasts independent of BMP-4 treatment. Because serum is reduced in both the mock and BMP-4 treated cells, *FOXC1* expression may induce pre-osteoblast differentiation instead of myotube formation which occurs in C2C12 myoblasts when resources for proliferation are low.

As expected from the 24 hours results, the increase in *Alpl* expression in untreated C2C12-pBABE-*FOXC1* cells is similar in pattern to what is shown in C2C12 cells undergoing BMP-induced osteoblast differentiation as represented in the literature (Figs 1.1, 3.14A, 3.15, 4.3, Table 4.1). Similar to *Alpl* expression, *Col1a1* expression shows a parabolic curve in untreated C2C12-pBABE-*FOXC1* cells (Figs 3.17A, 4.5), further suggesting *FOXC1* overexpression induces BMP-independent osteoblast differentiation. The increase in *Col1a1* expression occurs without a similar increase in *Runx2* or *Dlx3* expression in untreated C2C12-

pBABE-*FOXC1* cells (Figs 4.4, 4.5), further indicating a BMP-independent mechanism for osteoblast differentiation. Thus, FOXC1 may be acting either on the *Col1a1* promoter itself or it may be recruiting normal activating machinery like Runx2, Dlx3 or Dlx5 to the promoter with increased or constitutive binding stability. One prospective FOXC1 target sequence is present in the *Col1a1* proximal regulatory region.

Contrary to the trends seen in *Alpl* and *Col1a1* expression in C2C12-pBABE-*FOXC1* expression, *Opn* expression is decreased in untreated C2C12-pBABE-*FOXC1* cells relative to control cells (Fig 3.18). Because Opn is a component of the ECM, it follows that although increased FOXC1 production results in higher *Col1a1* expression and Alpl production, the ECM may not have the amount of Opn required for normal ECM mineralization and have structural abnormalities upon investigation. Therefore, a hypothesis may be that *FOXC1* induces many but not all genes required for osteoblast formation and ECM production, and therefore calcified bone resulting from increased FOXC1 production is not as strong or flexible as normal bone. Alternatively, ectopic *FOXC1* expression may be sufficient to begin osteoblast differentiation in myoblasts, but insufficient for differentiation to proceed past the pre-osteoblast stage; as increases in *Opn* and *Osx* are observed in maturing osteoblasts and are required for ECM mineralization (Fig 1.1, 4.5, Table 4.1).

The impact of stable *FOXC1* expression on *Alpl* expression suggests that FOXC1 may have an overlapping function with Wnt3a in that its expression induces Alpl production without the corresponding increase in BMP-SMAD signalling gene expression (Rawadi et al., 2003). However, the effect of stable *FOXC1* expression in the present research differs from the effect of Wnt3a expression with regards to *Col1a1* expression; Wnt3a expression does not affect *Col1a1* expression whereas stable *FOXC1* expression results in a dramatic increase. This suggests that although there may be some redundancy in Wnt3a and FOXC1 in osteoblast differentiation, their functions are distinct. More recent research highlighted that Wnt3a also induces BMP-2 production through canonical Catenin-B signalling, which may result in increased BMP-SMAD signalling (Zhang et al., 2013). The present research did not investigate the effect of *FOXC1* overexpression on BMP-2/4 production and stability within the cell though it is of interest for future research.

The changes in gene expression observed with and without BMP-4 treatment in C2C12-pBABE-*FOXC1* cells show the importance of proper gene regulation during periods of osteogenic differentiation and muscle and bone development. Hopkins et al. described the changes in *Foxc1* expression at different stages of osteoblast differentiation under BMP-4 induction (2015). They illustrated the importance of the cell's ability to increase and decrease *Foxc1* expression as differentiation proceeds from myoblast to mature osteoblast (Fig 4.6). This regulation occurs spatially as well, as seen in the differences in FOXC1 regulation between mouse myoblast cells undergoing osteoblast differentiation and developing mouse calvarial bones (Mirzayans et al., 2012; Sun et al., 2013). The present work shows that when myoblasts are unable to regulate *FOXC1* expression ectopic pre-osteoblast differentiation may occur.

4.3 Possible Clinical Significance

Although this research focuses on FOXC1's specific role in BMP-SMAD signalling, evidence for FOXC1 inducing BMP-independent osteoblastic differentiation was discovered. Also, considering C2C12 myoblasts are capable of osteoblast transdifferentiation when *FOXC1* is over expressed, this research may have interesting implications within clinical therapy. How

Figure 4.4

FOXC1 overexpression affects BMP-SMAD pathway gene expression in C2C12 myoblasts. Summary line graphs of results provided by Figures 3.11A, 3.12A, 3.13A, and 3.14A. Expression values are relative to expression of three control genes: *Hprt*, *Actin B*, and *Gapdh* at day 0 of BMP-4 treatment in C2C12-pBABE cells.

Figure 4.4

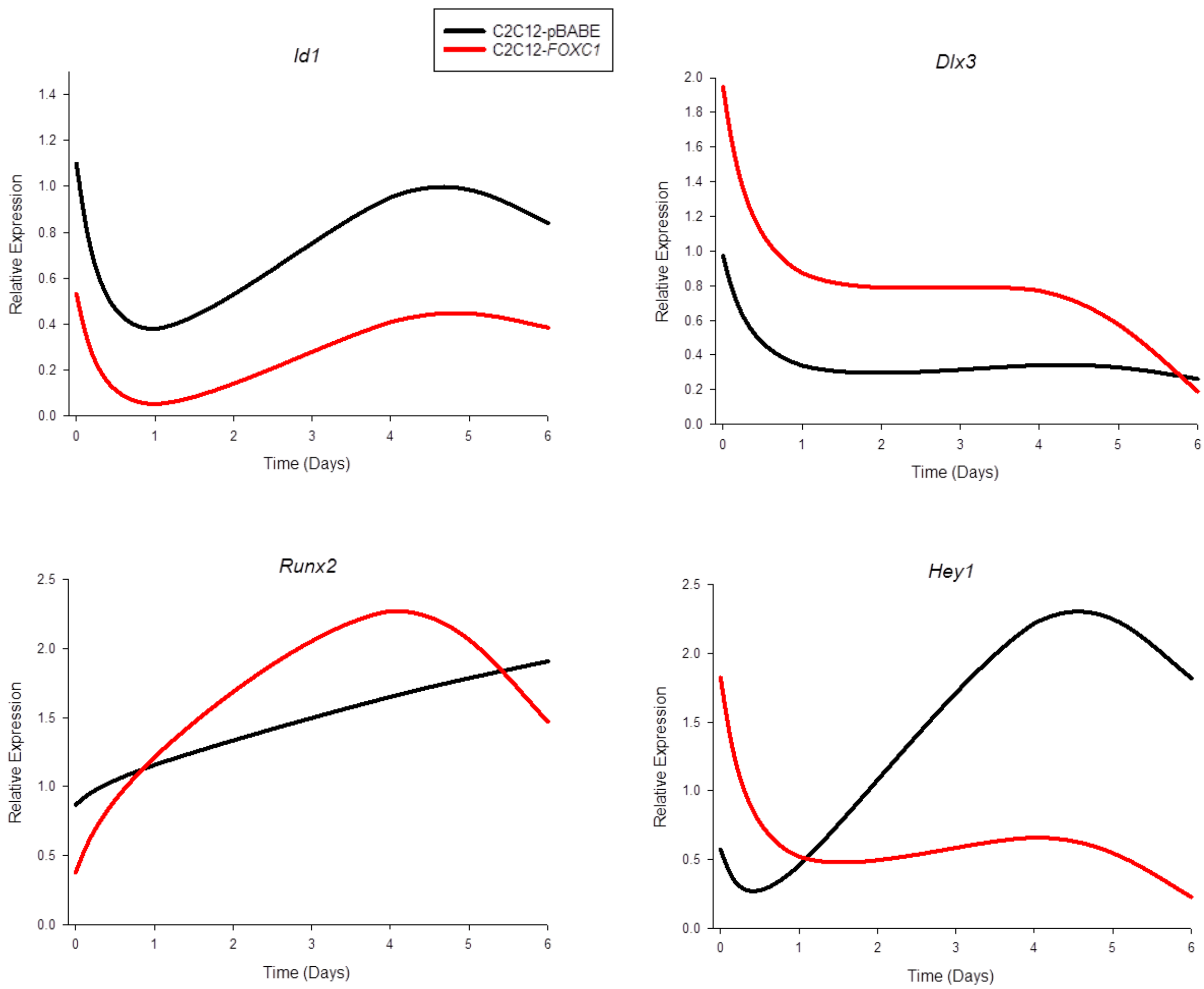
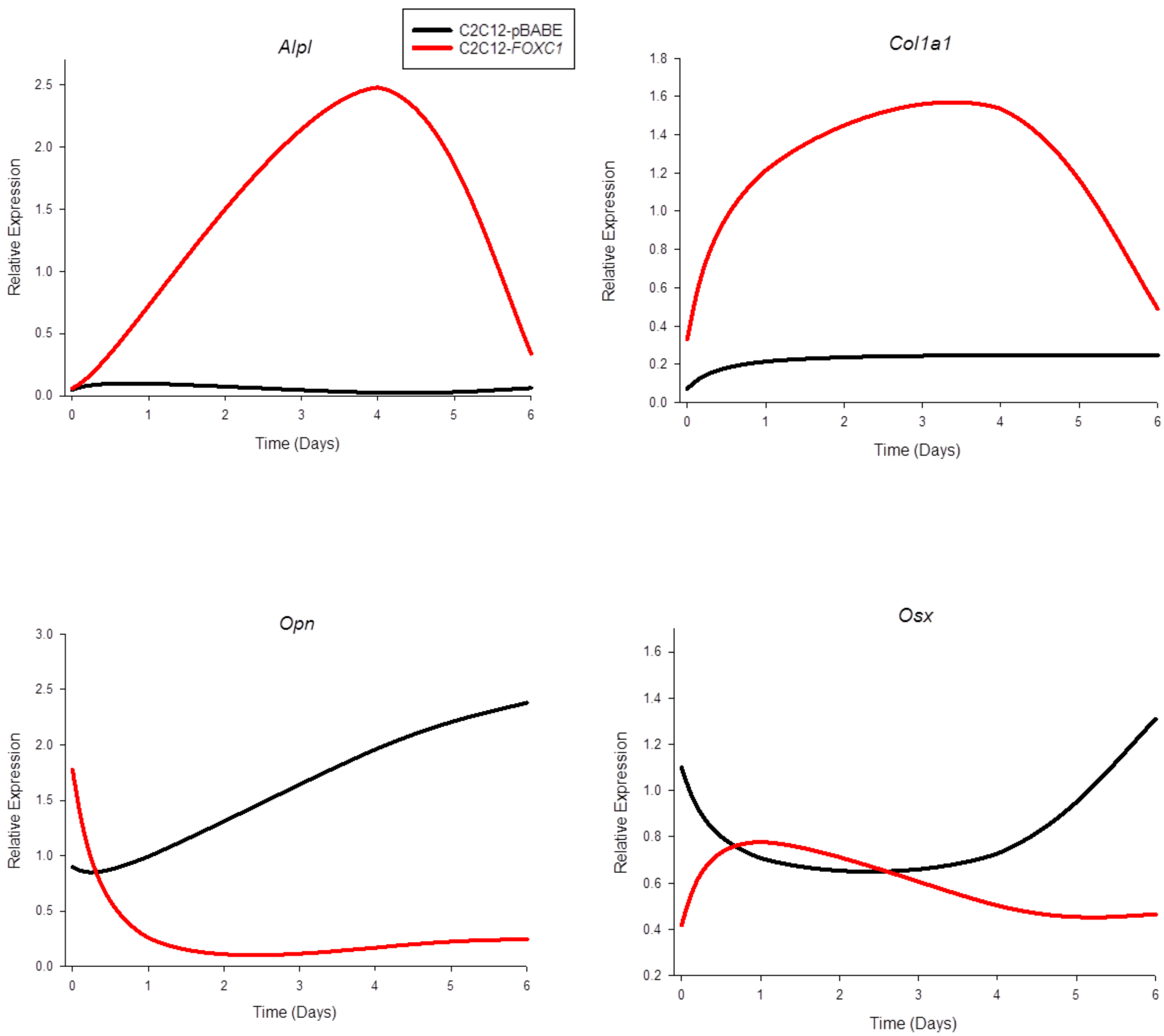

C2C12-pBABE
C2C12-*FOXC1*
Id1
Dlx3
Runx2
Hey1
Relative Expression
Time (Days)

Figure 4.5

FOXC1 overexpression affects osteoblast marker expression in C2C12 myoblasts. Summary line graphs of results provided by Figures 3.15A, 3.17A, 3.18A, and 3.19A. Expression values are relative to expression of three control genes: *Hprt*, *Actin B*, and *Gapdh* at day 0 of BMP-4 treatment in C2C12-pBABE cells.

Figure 4.5



C2C12-pBABE
C2C12-FOXC1
Alpl
Col1a1
Opn
Osx
Relative Expression
Time (Days)

Table 4.1

FOXC1 overexpression affects BMP signalling and osteoblast gene expression. Summary table depicting changes in gene expression under *FOXC1* overexpression examined in qPCR data. Arrows describe gene expression in C2C12-pBABE-*FOXC1* cells compared to C2C12-pBABE cells. Green upward arrow: gene is upregulated, red downward arrow: gene is downregulated, -: no change.

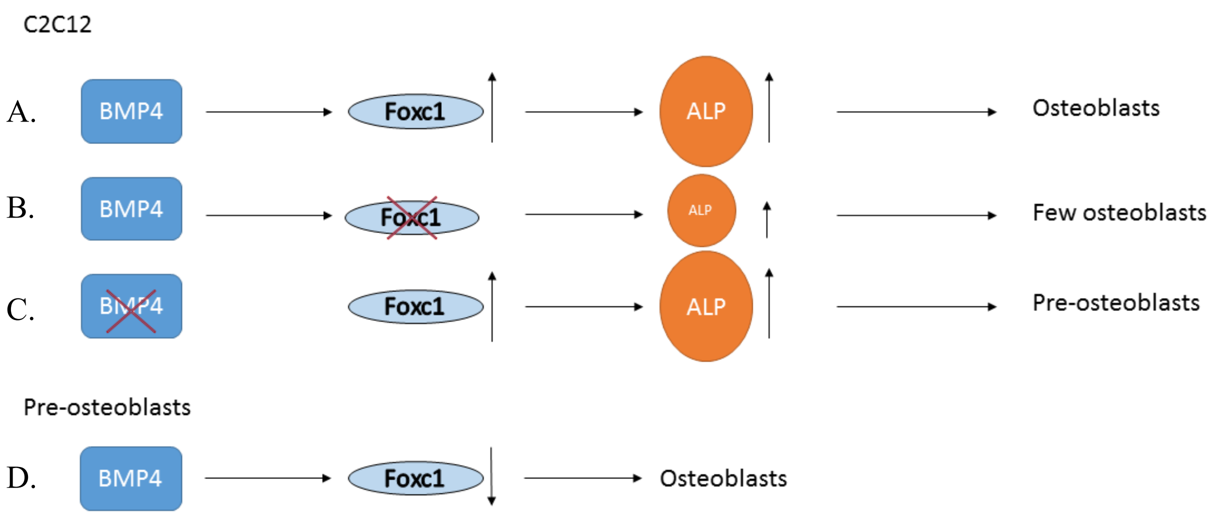
Table 4.1

Gene	- BMP4	+ BMP4
Id1	↓	↓
Dlx3	↑	↑
Runx2	-	↓ initially
Hey1	↓	-
Alpl	↑	delayed
Col1a1	↑	↑
Opn	↓	↑ late
Osx	↓ late	-

Figure 4.6

FOXC1 expression during and impact on osteoblast trans- and differentiation. Summary of the present work and previous work performed by Hopkins et al. (2015). **A.** BMP-4 treatment induces *Foxc1* expression during C2C12 transdifferentiation to osteoblasts. **B.** When Foxc1 is knocked down, BMP-4 induced transdifferentiation is not as efficient, and few osteoblasts result. **C.** An increase in *FOXC1* expression induces transdifferentiation independent of BMP-4, though C2C12 cells appear to hold in a pre-osteoblast state. **D.** In MC3T3 pre-osteoblasts, BMP-4 treatment leads to the decrease of *Foxc1* expression as cells differentiate to osteoblasts.

Figure 4.6


C2C12
A.
BMP4
Foxc1
ALP
Osteoblasts
B.
BMP4
Foxc1
ALP
Few osteoblasts
C.
BMP4
Foxc1
ALP
Pre-osteoblasts
Pre-osteoblasts
D.
BMP4
Foxc1
Osteoblasts

FOXC1 may impact muscle calcification will first be explored, followed by possibilities for improved bone formation in diseases of bone resorption like osteoporosis.

4.3.1 ***Muscle calcification and implications for FOXC1***

Calcification can occur in skeletal, cardiac and smooth muscle in response to disease and aging (Bessueille & Magne, 2015; Bostrom et al., 2011). Chronic Kidney Disease (CKD) and type II diabetes mellitus can present in comorbidity with calcification, and general atherosclerosis and aortic stenosis can be characterized by calcification of the inner lining of the blood vessels (Bessueille & Magne, 2015; Dweck et al., 2012; Paloian & Giachelli, 2014). Muscle calcification proceeds as cells differentiate to osteoblasts through the BMP-SMAD pathway (Bostrom et al., 2011; Nakagawa et al., 2010; Steitz et al., 2001). BMP-2/4 SMAD signalling unfolds canonically with RUNX2 and MSX2 expression inducing downstream targets such as *SP7*, *COL1A1*, and eventual *ALPL* expression. However, BMP induction of this process is not necessary, as tumor necrotic factor alpha (TNF-α) treatment results in ALPL production and calcification in vascular smooth muscle cells (vsmc), calvaria precursor cells, and human mesenchymal stem cell-derived osteoblasts independent of BMP-SMAD signalling (Ding et al., 2009; Gilbert et al., 2002; Lee et al., 2010). TNF-α is also shown to induce *MSX2* and *ALPL* expression, and inhibit *RUNX2* and *COL1A1* expression through pathway crosstalk with WNT and NF-KB signalling.

The present research may provide insight regarding TNF-α-mediated muscle cell calcification as *FOXC1* overexpression induces osteoblastic transdifferentiation in myoblasts independent of BMP-SMAD signalling. This suggestion is strengthened by TNF-α involvement in the expression of other FOX proteins in a variety of cell lines. In cervical cell line C-33A, TNF-α induces FOXO1 expression to result in increased apoptosis in cancer cells (Zhang et al., 2015). Similarly, in human fibroblasts, FOXO1 expression is increased while TNF-α inhibits TGF-β SMAD signalling; resulting in reduced fibroblast proliferation in a model for diabetic wound healing (Wang et al., 2014). However, in human mesenchymal stem cells, TNF-α treatment was recently shown to inhibit FOXO1 expression, exacerbating oxidative damage in an osteoporosis model (Liao et al., 2016). TNF-α activity can also be mediated by FOXO3a in MC3T3 cells to inhibit apoptosis (Bin et al., 2016). FOXO3a is phosphorylated by an activation of ERK5-AKT pathway, preventing its nuclear translocation. This in turn prevents activation of Bim and FasL expression and inhibits TNF-α-mediated apoptosis in these cells (Bin et al., 2016). Considering other forkhead proteins affect and are affected by TNF-α in a variety of cell types including mesenchymal, FOXC1 may be interacting with TNF-α to transdifferentiate C2C12 cells from myoblast to osteoblast independent of BMP-4. Also, the regulation of FOXO1a by FOXC1 resisting oxidative stress in ocular trabecular meshwork seen by Berry et al (2008) may carry over to other cells of mesenchymal origin where TNF-α and FOXC1 may cooperate to regulate FOXO1 expression.

4.3.2 *FOXC1 in osteoporosis*

Osteoporosis is a bone remodeling disease wherein bone resorption outpaces bone formation causes bone fragility. In other words, osteoclast formation and activity is greater than osteoblast formation and activity. Osteoporosis may arise from decreased estrogen production in post-menopausal women, decreased androgen and progesterone production as individuals age, comorbidity with other diseases or conditions, or as a side effect of unrelated treatment (Drake et al., 2015; Emkey & Epstein, 2014; Tella & Gallagher, 2014). Interestingly, TNF-α, which induces muscle calcification as described above, is also pathogenic in increasing the rate of bone loss resulting from low estrogen in mice (Ammann et al., 1997). Likewise, repressing TNF-α and Il-1 activity in post-menopausal women reduces their rate of bone loss (Charatcharoenwitthaya et al., 2007).

Treatment for osteoporosis is well-established, though new methods are constantly being developed. However, discoveries in the present research may further inform ongoing investigation regarding postmenopausal osteoporosis and its treatment. For example, current therapeutic strategies target estrogen or estrogen receptor treatments, osteoclast apoptosis, the reduction of osteoclast production, and the enhancement of osteoblast production (Drake et al., 2015; Tella & Gallagher, 2014). These therapies increase bone formation and limits resorption of developed bone.

Where traditional modes of bone homeostasis are disrupted, increased bone formation is shown to improve osteoporotic symptoms (Drake et al., 2015). Increasing osteoblast formation in precursor cells may improve the rate of bone formation in osteoporotic patients. The present research describes that when *FOXC1* is highly expressed in myoblasts, ectopic preosteoblast formation is possible. However, *FOXC1* expression is reduced in preosteoblasts as differentiation proceeds under BMP induction, but is essential to proper bone formation from early precursors and *in vivo* development (Fig 4.5) (Hopkins et al., 2015; Kume et al., 1998). Therefore, increased *FOXC1* expression in cortical and trabecular bone may improve the rate of osteoblast formation when combined with a treatment to continue maturation past preosteoblasts.

In addition to muscle calcification and osteoporosis research, there may be other benefits of FOXC1-mediated bone formation. Of particular interest to the author is the BMP-independent nature of FOXC1-mediated osteoblast transdifferentiation. In individuals with mutations affecting BMP-SMAD signalling, targeted *FOXC1* upregulation may ameliorate some bone-related symptoms. For example, cleidocranial dysplasia is a disease resulting from mutations to *RUNX2*, which affects the proper formation and growth rate of bone (Guo et al., 2015). Inducing bone formation independent of BMP-SMAD signalling would bypass the mutation in *RUNX2* and may improve symptoms in patients. *FOXC1* expression may induce bone formation in a model for cleidocranial dysplasia, however there would be significant complications in ensuring proper bone formation through targeted increases in *FOXC1* expression, though with advances in medical and genetic research the challenges may be overcome.

4.4 Future Directions

The present research provides findings that encourage further research. Of immediate interest is the mechanism through which FOXC1 impacts BMP-SMAD signalling, particularly *Id1* expression. The author briefly undertook co-immunoprecipitation (CoIP) experiments to determine if FOXC1 interacts with either SMAD-4 or BMP-2/4 receptor SMADs 1 or 5. The author was unable to optimize the experiment for SMAD-4 interaction with SMAD-5 as a positive control for the experiment and findings produced concurrent with CoIP optimization resulted in other questions being investigated. Likewise, chromatin IPs (ChIP) were briefly undertaken to determine if FOXC1 localizes to the *Id1* proximal regulatory region, and if stable FOXC1 production results in a decreased ability for SMADs to localize to the *Id1* promoter region. Once again, the experiments were unsuccessful as the author was unable to identify SMAD protein binding to the *Id1* promoter. These experiments will be attempted next testing several possible functions of FOXC1 at the *Id1* promoter: FOXC1 may bind the proximal regulatory region and through chromatin remodeling may prevent SMAD complex binding its target; FOXC1 may bind the proximal regulatory region and through chromatin remodeling, may bind SMAD complex and change its function from activating transcription to preventing it; FOXC1 may bind the SMAD complex and sequester it away from its target on the *Id1* promoter. However, as *FOXC1* expression during bone formation is context sensitive as described by Sun et al (2013) and Mirzayans et al (2012), its effect on *Id1* expression may be as well. Thus, the interaction may occur in true biological systems, though not in the models used. Thus if no definitive answers are discovered regarding its interaction in the BMP-SMAD pathway, GST-pulldown assays can be implemented to determine if FOXC1 is capable of binding activated pSMAD-1/5/9. As GST-pulldowns showing FOXC1 is capable of binding SMAD-4 has previously been shown, there is no need for further GST-pulldowns except as a positive control (Fujita et al., 2006).

Also of interest is the findings that the N-AD, IHD and FHD have important roles in FOXC1 inhibition of *Id1* transcription. CoIPs and ChIPs may provide information as to the nature of any interaction discovered in the experiments suggested above. Previous work by Han et al (2015) indicated that both the N-AD and FHD of FOXC1 is important for effective binding with Gli2. Thus in this case, both the N-AD and a functional FHD may be required for interaction with SMADs. Alternatively, Berry et al (2006) show that the N-AD is not required for binding with actin filamin A protein, suggesting that the regions required for FOXC1 protein-protein interactions change with the transcription factor being bound. Therefore, each region should be investigated for a role in any FOXC1-SMAD interaction discovered.

The different responses of genes such as *Id1* and *Hey1* may be an effect of the cell’s ability to respond to BMP treatment and serum starvation. As stated earlier, the passage number of the cells were unknown and thus cell behavior and health were closely monitored visually. However, the cell's ability to respond to BMP may have been affected if the cells appeared similar to previous passages or not. This may have affected the results I observed. FOXC1 levels may also be affected simply by changing media to a serum reduced form. Future investigation should ensure passage number and cellular health throughout the experiments. Also, BMP stability in the cells and media should be monitored throughout the time course experiment, and an additional experiment with consistent concentrations of BMP in the media throughout the time course may provide further information regarding FOXC1's involvement in BMP-induced and ectopic osteoblast differentiation. Finally, the monitoring of FOXC1 levels in cells growing in serum reduced and normal (10% FBS) media is recommended.

The present research's finding that *FOXC1* overexpression induces osteoblast differentiation in C2C12 myoblasts offers possibilities for further investigation in different models of osteoblast differentiation and muscle calcification. The next step is to repeat the present research in mouse embryonic stem (ES) cells to determine if FOXC1 is capable of inducing bone formation in stem cells as well as muscle precursor cells. Further investigation using human and mouse muscle stem cells will test the hypothesis FOXC1 induces muscle calcification and osteoblast differentiation in mesenchymal cells. Testing *FOXC1* overexpression in human vascular muscle cells will examine the possibility that FOXC1 is an important player in muscle calcification. Also, testing Type II diabetes mellitus, atherosclerosis, CKD, and aortic stenosis patients for increased FOXC1 production *in vivo* will help us understand the role FOXC1 may play in muscle calcification as a symptom of disease or aging.

The ability of TNF-α to induce muscle calcification independent of BMP-SMAD signalling induction demonstrates a possible overlap or relationship with FOXC1 in light of the present research (Ding et al., 2009; Gilbert et al., 2002; Lee et al., 2010). Vsmcs may be used as a model for investigation into this possible relationship, and testing for increased *FOXC1* expression in response to TNF-α and *vice versa* may illustrate a relationship between the two proteins in muscle calcification previously unknown.

Recently, microRNA (miRNA or miR) research has indicated FOXC1 production is regulated by miRNAs in biological processes. For example, miR-639 targets FOXC1 3' untranslated region (3'-UTR) to inhibit FOXC1-mediated epithelial to mesenchymal cellular transitions (EMT) (Lin et al., 2014). Another miRNA, miR-138-5p was recently shown to reduce FOXC1 production by also targeting FOXC1 3'-UTR to inhibit pancreatic cancer growth and metastasis (Yu et al., 2015). MicroRNAs may be capable of inhibiting FOXC1 production in muscle calcification models as well. Future studies to investigate the impact miRNA treatment will have on FOXC1-induced C2C12 calcification may show promising results.

FOXC1 has been known as an important factor in proper bone formation for more than a decade; however, FOXC1's role in osteogenesis is largely unknown. The present research identifies functional regions of FOXC1 important for its inhibitory effect on *Id1* expression. Also, this work shows FOXC1 induces preosteoblast differentiation in C2C12 cells independent of BMP-signalling and that *FOXC1* overexpression in cells treated with BMP-4 decreases the efficiency of transdifferentiation seen in wildtype or control C2C12 cells. The present work contributes to the field's understanding of how *FOXC1* expression affects osteogenic differentiation (Fig 4.5). These findings suggest FOXC1 may be an important inducer of muscle calcification previously undiscovered. Further investigation is required to discover the mechanism of FOXC1 action on *Id1* expression and myoblast-osteoblast transdifferentiation.

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