INVESTIGATION OF THE ROLE OF INTERDIGIT BMP SIGNALING IN PROGRAMMED CELL DEATH AND MOUSE LIMB DEVELOPMENT

by

Maria Mateeva Kaltcheva

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

July 2015

© 2015 Maria M. Kaltcheva

All Rights Reserved
ABSTRACT

Shaping of the embryonic limb involves many processes including growth, differentiation, and programmed cell death (PCD). Furthermore, these processes integrate complex information from multiple signaling cascades such as the BMP and FGF pathways.

Previous work shows that BMP signaling regulates interdigit (ID) PCD indirectly by affecting expression of Fgfs, which encode cell survival factors secreted from the apical ectodermal ridge. Nevertheless, this indirect model does not exclude a direct role for BMPs in PCD. Due to lack of genetic evidence, it is still unclear whether ID BMPs directly trigger PCD.

To test whether BMPs act as direct triggers of ID PCD, the gene encoding receptor BMPR1A was inactivated within the ID tissue with Osr1-Cre. The resulting mutants show a decrease of ID PCD. To test redundancy between BMPR1A and BMPR1B, ID-specific Bmpr1a was inactivated in a Bmpr1b null background. This compound mutant has a further decrease in ID PCD. Analysis of BMPR1A mutants reveals a reduction in ID BMP signaling as observed through a decrease in Msx2 expression and phosphorylated SMAD 1 and 5 levels. Furthermore, a decrease in ID Dkk1 expression and a lack of ID webbing in Osr1-Cre; Smad4 mutants suggests both SMAD independent and dependent components in the regulation of ID PCD.

During this analysis, a potential role of the ID in digit formation was serendipitously discovered. Bmpr1b null mice have brachydactyly, which is completely rescued in digit one with the ID-specific inactivation of Bmpr1a. Furthermore, preliminary analysis of mice with both Bmp7 and Bmpr1a inactivated in the ID of
*Bmpr1b* mutants suggests that brachydactyly is recapitulated and ID BMP7 secretion is necessary to rescue digit development.

To fully understand the role of ID BMP signaling on normal limb development, ID *Bmp2, 4, and 7* were inactivated in various combinations with Osr1-Cre. Analysis of these mutants reveals that BMP7 is necessary for ID BMP signaling and PCD during normal development, while BMP2 and BMP4 play redundant roles in ID PCD regulation.

This work establishes that BMP signaling directly regulates ID PCD and that ID BMP secretion can regulate digit development.

**Thesis Committee:**

Dr. Mark Lewandoski  
Advisor, Cancer and Developmental Biology Lab, NCI/NIH  
Dr. Chen-Ming Fan  
Carnegie Institution -Department of Embryology and CMDB/JHU  
Dr. Susan Mackem  
Cancer and Developmental Biology Lab, NCI/NIH  
Dr. Yingzi Yang  
Harvard School of Dental Medicine  
Dr. Marnie Halpern  
Carnegie Institution -Department of Embryology and CMDB/JHU

**Readers:**

Drs. Mark Lewandoski and Chen-Ming Fan
ACKNOWLEDGEMENTS

It has been an absolute pleasure being a part of and conducting my thesis work in Dr. Mark Lewandoski’s laboratory. In addition to being a wonderful mentor and role model, Dr. Lewandoski’s intellectual input and scientific inquiry have been inspirational throughout my doctorate work.

I would like to express my very great appreciation to everyone within all four sections of the Cancer and Developmental Biology Laboratory and the wonderfully collaborative environment they have created. In particular, Dr. Matthew Anderson, who has been like a second mentor to me during my graduate studies.

I would like to acknowledge the members of my thesis committee, Dr. Chen-Ming Fan, Dr. Susan Mackem, Dr. Yingzi Yang, and Dr. Marnie Halpern for their scientific guidance and support throughout my graduate studies.

I am very grateful to all of the investigators who have allowed us to use the mice they generated: Dr. Gail Martin for the Osr1-Cre transgenic mice, Dr. Yuji Mishina for the Bmpr1a flox and null mice, Dr. Karen Lyons for the Bmpr1b null mice, Dr. Brian Harfe for the Bmp2, 4, and 7 flox mice, and Dr. Chuxia Deng for the Smad4 flox mice.

Finally, I would like to thank my incredible support system of family, friends, classmates, and colleagues, both in the United States and Bulgaria. Special thanks to Dr. Prashob Porayette who mentored me during my undergraduate studies at UW – Madison and introduced to me the wonders of developmental biology. I am particularly grateful to Geoffrey Vargish for his constant encouragement, never-ending support, and contagious scientific enthusiasm. To my wonderful and amazing mom and dad – Цуня!
# TABLE OF CONTENTS

## Chapter 1. Introduction

1.1 Universality of this work and relevance of programmed cell death .................. 2
1.2 Limb development overview ........................................................................... 3
1.3 Overview of Bone Morphogenetic Protein signal transduction ....................... 4
1.4 Previous evidence for BMPs as direct effectors of programmed cell death ...... 5
1.5 Retinoic acid as an interdigit cell death inducer ............................................. 5
1.6 Evidence for indirect role of BMPs as cell death effectors through AER-FGFs .. 6
1.7 Role of senescence in limb morphogenesis and interdigit programmed cell death ................................................................................................................................................. 8
1.8 Vascular patterning in interdigit programmed cell death regulation .............. 9
1.9 Current model for interdigit programmed cell death regulation and outstanding questions ................................................................................................................................................. 10
1.10 Interdigit tissue as a signaling center for digit formation and identity .......... 11

## Chapter 2. Methods

2.1 Ethics statement ............................................................................................... 15
2.2 Tables of genetic crosses used within this thesis ............................................ 15
2.3 Mouse line maintenance and genotyping ....................................................... 19
2.4 LysoTracker Red assay for cell death detection ............................................. 21
2.5 X-Gal staining for β-galactosidase activity and sectioning .............................. 21
2.6 Whole mount in situ hybridization ................................................................. 22
2.7 Whole mount immunolabeling ......................................................................... 22
2.8 Senescence associated β-galactosidase assay ............................................... 23
Chapter 3. Evaluation of programmed cell death initiation and progression during normal mouse limb development and relative to Osr1-Cre activity

3.1 Characterizing programmed cell death initiation and progression during limb development via LysoTracker Red

3.2 Characterizing Osr1-Cre activity and timing relative to mouse limb development

3.3 Changes in AER-Fgf8 mRNA expression

3.4 Characterizing interdigit BMP activity via changes in Msx2 mRNA expression during programmed cell death

3.5 Summary

3.6 Discussion

Chapter 4. Interdigit BMP 7 ligand secretion is necessary for programmed cell death during normal development

4.1 Interdigit Bmp 2, 4, 7 expression domain during normal development and following inactivation with Osr1-Cre
4.2 Examining necessity and redundancy of interdigit BMP 2, 4, 7 in programmed cell death regulation.................................................................40
4.3 Analysis of Bmp 2, 4, 7 mutants.................................................................53
4.4 Summary.................................................................................................59
4.5 Discussion.................................................................................................59

Chapter 5. BMP signaling directly regulates interdigit programmed cell death

5.1 Interdigit BMP signaling through BMPR1A is necessary for programmed cell death.................................................................................62
5.2 BMPR1B plays a redundant role in apoptotic interdigit programmed cell death......................................................................................63
5.3 Analysis of the cell autonomous ID Bmpr1 inactivation ........................................70
5.4 Role of interdigit BMP receptors in senescence, vascular patterning, and levels of reactive oxygen species....................................................71
5.5 Potential role of SMAD independent signaling in the regulation of interdigit programmed cell death.................................................................75
5.6 Inactivation of Bmpr1a in the AER and interdigit further decreases interdigit programmed cell death.............................................................79
5.7 Summary..................................................................................................81
5.8 Discussion.................................................................................................82

Chapter 6. Evaluating the role of the limb interdigit tissue as a signaling center necessary for skeletogenesis and digit outgrowth during normal development

6.1 Interdigit Bmp 2, 4, 7 inactivation does not affect digit formation and skeletogenesis.............................................................................86
6.2 Interdigit $Bmpr1a$ inactivation in $Bmpr1b$ null mutants rescues digit one outgrowth.................................................................89

6.3 Histological examination of digit one rescue in Osr1-Cre; $Bmpr1a^{flox/null}; Bmpr1b^{null/null}$ mutants at P0.5.................................................................94

6.4 Gdf5 expression is not rescued in the proximal phalanx of Osr1-Cre; $Bmpr1a^{flox/null}; Bmpr1b^{null/null}$ embryos.................................................................97

6.5 Abolishing apoptotic cell death does not rescue brachydactyly in $Bmpr1b$ null mutants.................................................................100

6.6 Interdigit $Bmp7$ is necessary for rescue of digit one development in Osr1-Cre; $Bmpr1a^{flox/null}; Bmpr1b^{null/null}$ mice.................................................................103

6.7 Summary.................................................................110

6.8 Discussion.................................................................110

Chapter 7. General discussion and future directions ...............................113

Appendix: Examining interdigit gene expression in Msx2-Cre $Bmpr1a$ mutants via microarray analysis .................................................................120

References.................................................................124

Curriculum Vita.................................................................133
LIST OF FIGURES

CHAPTER 1. Introduction

Figure 1. Current model of interdigit programmed cell death regulation and outstanding questions .........................................................12

CHAPTER 3. Evaluation of programmed cell death initiation and progression during normal mouse limb development and relative to Osr1-Cre activity

Figure 2. Characterization of programmed cell death initiation and progression during normal mouse limb development and relative to Osr1-Cre activity...........32

Figure 3. Characterization of Osr1-Cre activity in the developing mouse autopod....34

Figure 4. Variability of Osr1-Cre deletion efficiency.........................................................35

CHAPTER 4. Interdigit BMP 7 ligand secretion is necessary for programmed cell death during normal development

Figure 5. Individual inactivation of Bmp2, 4, and 7 with Osr1-Cre reveals that ID BMP7 is necessary for PCD during normal development.................43

Figure 6. Combinatorial inactivation of ID Bmp ligands reveals redundancies in ID PCD.................................................................46

Figure 7. Bmp2 inactivation sensitizes the ID mesenchyme to Bmp7 perturbation….49

Figure 8. Simultaneous inactivation of Bmp2, 4, and 7 with Osr1-Cre.................51

Figure 9. ID Bmp2, 4, 7 inactivation results in a decrease of ID PCD without affecting AER-Fgf8 expression or retinoic acid signaling.........................54
Figure 10. A decrease in cleaved Caspase 3, Msx2, and phosphorylated SMAD 1 and 5 is observed in Osr1-Cre Bmp2, 4, and 7 mutants.........................57

CHAPTER 5. BMP signaling directly regulates interdigit programmed cell death

Figure 11. ID Bmpr1a is necessary for apoptotic PCD during limb development while Bmpr1b plays a redundant role...............................................................66

Figure 12. Osr1-Cre Bmpr1 inactivation results in a decrease of Msx2 and pSMAD 1, 5 without affecting AER-Fgf8 and retinoic acid signaling ..................68

Figure 13. A decrease in senescence and reactive oxygen species is observed upon ID Bmpr1 inactivation..........................................................73

Figure 14. Normal vascular patterning is observed in Osr1-Cre; Bmpr1a$^{\text{flox/null}}$ syndactyous mutants .................................................................74

Figure 15. A decrease in ID Dkk1 expression in Osr1-Cre; Bmpr1a$^{\text{flox/null}}$ mutants suggests involvement of SMAD independent BMP signaling.............77

Figure 16. Lack of syndactyly in Osr1-Cre; Smad4$^{\text{flox/null}}$ mice suggest involvement of SMAD independent regulation of ID BMP signaling............78

Figure 17. Inactivation of Bmpr1a with Msx2-Cre and Osr1-Cre further decreases ID PCD............................................................80

CHAPTER 6. Evaluating the role of the interdigit tissue as a signaling center necessary for skeletogenesis and digit outgrowth during normal limb development

Figure 18. Bmp2, 4, and 7 inactivation with Osr1-Cre does not result in any apparent skeletal abnormalities. .........................................................88
Figure 19. Interdigit Bmpr1a inactivation in Bmpr1b null mutants rescues digit one outgrowth.................................................................92

Figure 20. Histological analysis of Osr1-Cre; Bmpr1a^{flox/null}, Bmpr1b^{null/null} neonates.................................................................96

Figure 21. Characterization of Gdf5 expression in Osr1-Cre; Bmpr1a^{flox/null}, Bmpr1b^{null/null} embryos.........................................................99

Figure 22. Abolishing apoptotic cell death does not rescue brachydactyly in Bmpr1b null mutants..............................................................102

Figure 23. Removing one copy of the BMP inhibitor, Noggin, does not affect digit one rescue in Osr1-Cre; Bmpr1a^{flox/null};Bmpr1b^{null/null} mice.............106

Figure 24. Inactivating ID Bmp7 causes brachydactyly in digit one of Osr1-Cre; Bmpr1a^{flox/null};Bmpr1b^{null/null} mice.............................................108

CHAPTER 7. Summary and general discussion

Figure 25. Updated model of interdigit programmed cell death regulation during normal development....................................................118

APPENDIX.

Figure 26. Transcriptome analysis of genes that are at least two-fold upregulated in interdigit one of Msx2-Cre; Bmpr1a^{flox/null} E13.5 embryos..............121

Figure 27. Transcriptome analysis of genes that are at least two-fold downregulated in interdigit one of Msx2-Cre; Bmpr1a^{flox/null} E13.5 embryos..............123
LIST OF TABLES

Table 1. Chapter 3 Genetic Crosses ................................................................. 15
Table 2. Chapter 4 Genetic Crosses ................................................................. 16
Table 3. Chapter 5 Genetic Crosses ................................................................. 17
Table 4. Chapter 6 Genetic Crosses ................................................................. 18
Table 5. List of relevant PCR reactions with corresponding primer sequences .... 20
CHAPTER 1. INTRODUCTION
1.1 Universality of this work and relevance of programmed cell death

The transformation from a single cell to a fully functional organism involves many dynamic processes that guide basic cellular function such as proliferation, migration, differentiation, and programmed cell death (PCD). Furthermore, during embryogenesis the integration of complex signal transduction pathways from various developing tissue is required for normal development. Limb development has been a robust model for over 50 years to study complex cellular processes such as PCD and the basic steps of skeletal development\(^1\)\(^-\)\(^4\). In particular, PCD is a widely occurring mechanism necessary for sculpting the developing limb and for the removal of tissues that play a transitory role during embryogenesis\(^5\)\(^,\)\(^6\). Many classical studies on limb development have been performed using chick embryos\(^4\)\(^,\)\(^7\)\(^-\)\(^{11}\). However, due to the available tools for its genetic manipulation and its high degree of physiological similarity with humans, the organism \textit{Mus musculus} (mouse) is an extremely useful and robust model to further investigate the complex processes of PCD and limb development\(^12\)\(^,\)\(^13\).

Given the complexities of limb development, it is understandable that congenital limb defects are some of the most prevalent birth malformations (1 in 500 to 1 in 1000 human live births)\(^14\). Of these, syndactyly, the retention of tissue between the forming digits, is the most common, with an incidence of 1 in 2000 to 1 in 3000 human live births\(^15\)\(^-\)\(^17\). Furthermore, clinical studies have shown that, in some of the malformations, signaling cascades known to regulate PCD are involved\(^18\). A better understanding of PCD during normal development is necessary to elucidate these pathogenic states and give insight into pleiotropic effects of gene regulation during embryogenesis\(^14\).
1.2 Limb development overview

Limbs begin as buds of the lateral plate mesoderm that emerge from the trunk perpendicular to the developing body axis\(^4\). The forelimb and the hindlimb develop asynchronously with the forelimb bud emerging at approximately mouse embryonic day (E) 9.5 and the hindlimb about twelve hours later. The mesenchymal cells are jacketed by ectoderm that migrates on the ventral part of the limb to the bud apex, forming a pseudo-stratified structure known as the apical ectodermal ridge (AER)\(^{19-22}\). The AER is necessary for proper limb outgrowth and manipulations in chick embryos have shown that its removal results in limb truncations\(^4,11\). In particular, Fibroblast Growth Factor (FGF) secretion from the AER signals to the underlying mesenchyme and is necessary for limb bud outgrowth\(^{23-28}\). The four known Fgfs expressed in the AER during limb development are Fgf8, 4, 9, and 17. Of these, Fgf8 is expressed in the greatest anterior – posterior range throughout the AER and the longest time throughout limb development\(^{29,30}\). Furthermore, within the limb bud mesenchyme are progenitors of the limb skeletal elements and tendons\(^{31-33}\). As limb outgrowth proceeds, spatio-temporal information is integrated along three axes: anterior – posterior (thumb to pinky), proximal – distal (shoulder to fingertips), and dorsal – ventral (knuckles to palm)\(^4,34\). During development the limb bud grows and differentiates into three distinct parts of the mature limb: stylopod (humerus/femur), zeugopod (ulna and radius/tibia and fibula), and, most distally, the autopod (hand/feet)\(^35\). At approximately E12.5, the autopod consists of Sox9 positive condensed mesenchyme that gives rise to the future skeletal elements of the digits\(^36,37\). These digital rays are separated by Sox9 negative interdigit (ID) mesenchyme\(^36,37\). The ID mesenchyme is ultimately removed by E14.5 through the
genetically regulated process of PCD, leading to the separation and individualization of the digits. However, while transiently present during autopod development, evidence suggests that the ID could be an instructive signaling center necessary for proper digit formation and identity. Perturbation of the process of PCD leads to the retention of the ID mesenchyme and webbing between the digits of the adult, a phenotype referred to as soft tissue syndactyly. Even though there has been extensive work investigating which signaling cascades play a role in the regulation of PCD, it is still unclear whether bone morphogenetic proteins (BMPs) are direct regulators of ID PCD.

1.3 Overview of Bone Morphogenetic Protein signal transduction

The BMP signal transduction pathway is part of the greater Transforming Growth Factor β (TGFβ) superfamily. In the limb ID mesenchyme, BMP signal transduction is initiated when a BMP ligand dimer binds to and subsequently leads to the phosphorylation and activation of three Ser/Thr kinase type BMP receptors: BMPRII, BMPRIA, and BMPRIB. The BMP ligand dimer first binds to the type II receptors, which recruit and activate a type I receptor dimer via phosphorylation on Ser/Thr residues. In the case of canonical BMP signaling, receptor regulated SMADs (R-SMADs) 1, 5, and 8 are recruited and activated by another phosphorylation event. The R-SMADs recruit and bind co-SMAD4, which translocates this complex to the nucleus and regulates BMP transcriptional targets, such as Msx2, involved in limb development. Although BMP receptors II, IA, IB and the R-SMADs 1, 5, and 8 are specific to canonical BMP signaling, the co-SMAD4 is also an integral part in the TGFβ signaling cascade. Furthermore, developmental processes, such as PCD and
chondrogenesis, likely involve synergistic regulation through both BMP/SMAD dependent and independent cascades \(^{47,49,50}\).

1.4 Previous evidence for BMPs as direct effectors of programmed cell death

BMP signaling is necessary for proper limb morphogenesis throughout development, such as digit identity specification, anterior-posterior and dorsal-ventral patterning, and regulation of the apical ectodermal ridge \(^{2,3,8,41,42}\). BMP signaling has also been implicated as a direct effector and regulator of ID PCD, necessary for the removal of the ID mesenchyme and digit separation \(^{39,40,51}\). The expression pattern of BMP ligands suggests that they likely play a role in initiation of ID PCD. Bmp ligands 2, 4, 5, and 7 have different but overlapping expression patterns in the ID region during limb development that correlate with the initiation of ID PCD \(^{10,34,42,50,52-54}\). Furthermore, ectopic application of the BMP ligands 2, 4, 5, and 7 promote the initiation of apoptotic cell death and accelerate the regression of the ID mesenchyme \(^{44,50,51,55,56}\). Conversely, the BMP antagonists Noggin or Gremlin have been shown, either by exogenous application or transgenic overexpression in the ID mesenchyme, to inhibit PCD \(^{57,58}\). Finally, exogenous expression of a dominant-negative BMP receptor in chick hind limbs through retroviral expression inhibited PCD and led to the retention of ID mesenchyme \(^{59,60}\). Together, these studies and the normal expression domains of key BMP related genes suggest that BMPs directly regulate ID PCD.

1.5 Retinoic acid as an interdigit cell death inducer

Retinoic acid (RA) is a metabolite produced from Vitamin A and is necessary for
normal embryogenesis and development. During early embryogenesis, the levels of RA are regulated through its synthesis by Raldh2 and through the metabolite’s inactivation via the Cyp26 family of enzymes. Early developmental experiments have implicated retinoic acid as a positive regulator of cell death. For example, an increase in RA levels during limb bud outgrowth leads to distal limb truncations and its exogenous application just prior to the initiation of ID PCD has been shown to accelerate the process of ID regression. In contrast, exogenous application of RA receptor antagonists decreases ID PCD. These changes in PCD due to increase or decrease of RA levels also affect mRNA expression of the pro-apoptotic factor Bax in the distal mesenchyme. In the presence of exogenous RA there is an increase in Bax expression, whereas application of RA receptor antagonists causes a decrease in Bax expression. Furthermore, expression of the RA receptor Rarβ is restricted to the ID mesenchyme, suggesting that it could play a role in the regulation of PCD. In fact, retention of ID tissue is observed in Rarβnull/null; Rarγnull/null double mutants. Exogenous RA application also correlates with a decrease in Fgfr1 expression and Erk1/2 phosphorylation, components of the FGF pathway.

1.6 Evidence for indirect role of BMPs as cell death effectors through AER-FGFs

During normal embryonic development, Fgf8 is expressed throughout the AER and signals to the underlying mesenchyme through Fgfr1. However, around the time of ID PCD initiation, Fgf8 expression overlying the ID mesenchyme is down regulated and becomes restricted to the AER above the digital rays. Furthermore, exogenous application of beads soaked with FGF protein (e.g. FGF2, FGF4, or FGF8) inhibits ID PCD from occurring. Alternatively, inhibiting FGF signal transduction
results in an increase of cell death in the ID. For example, in limb cultures with the FGF receptor inhibitor, SU5402, an increase in cell death is observed\textsuperscript{43}. Phosphorylation of the MAP kinases ERK1/2 has been associated with FGF activity from the AER and a similar increase in cell death is observed upon culturing limbs with the MAP kinase inhibitor UO126\textsuperscript{43,69}. Taken together, these data suggest that, during normal development, FGFs secreted from the AER are involved in the regulation of PCD of the underlying mesenchyme by acting as survival factors.

Several mouse mutants also implicate the involvement of FGFs from the AER in the regulation of PCD in the underlying mesenchyme\textsuperscript{42,70-72}. Furthermore, some of these studies show that BMP signaling to the AER directly regulates AER-Fgf expression and thus indirectly regulates ID PCD\textsuperscript{42,70,71}. Tissue-specific inactivation of Bmp2 and Bmp4 with the AER-specific Msx2-Cre results in a syndactylosphenotype due to an abnormal spatio-temporal upregulation and expansion of Fgf4 and Fgf8 in the AER\textsuperscript{70,71}. The indirect regulation AER-Fgf expression via BMPs was even more clearly shown, previously in the Lewandoski laboratory, through the AER tissue-specific inactivation of Bmpr1a with Msx2-Cre\textsuperscript{42}. In this study, AER specific Bmpr1a inactivation led to a dorso-ventrally broader AER and the abnormal retention of Fgf4 and Fgf8 expression above the ID mesenchyme through E14.5\textsuperscript{42}. These Msx2-Cre; Bmpr1a\textsuperscript{flox/null} mutants do have a syndactylosphenotype without an observable change in ID BMP signaling\textsuperscript{42}. For example, there was no observable difference in mRNA expression between controls and mutants when examining several aspects of the BMP signaling cascade such as the Bmp2, 4, and 7 ligands, the downstream BMP targets Msx1 and Msx2, and the BMP inhibitor Gremlin\textsuperscript{42}. Concurrent AER inactivation of Fgf4 and Fgf8 in the Msx2-Cre;
Bmpr1a\textsuperscript{flax/null} mutants rescues the syndactylous phenotypes and demonstrates that the decrease in ID PCD is due to the spatio-temporal increase in AER-Fgf expression\textsuperscript{42}.

This revised model of ID PCD regulation in the developing limb questions the validity of the existing paradigm that BMPs act as direct cell death effectors in the regulation of ID PCD. In fact, in several previous studies implicating BMPs as direct effectors of ID PCD, AER-Fgf expression was also up-regulated and expression abnormally persisted in cells overlying the ID\textsuperscript{48,57,70,73}. Furthermore, initiation of ID PCD does not temporally correlate with the onset of BMP ligand and BMP target gene expression\textsuperscript{43}. Thus, in light of BMPs’ indirect role in ID PCD through regulating AER-Fgfs, it is still unclear whether ID BMP signaling directly regulates PCD in the ID mesenchyme.

1.7 Role of senescence in limb morphogenesis and interdigit programmed cell death

Cellular senescence has generally been associated with oncogenic and pathogenic states. However, there is recent evidence that this form of cell cycle arrest could be part of normal embryogenesis necessary for proper organ morphogenesis, such as the formation of the AER\textsuperscript{74}. One hypothesis is that senescence could be part of a normal developmental program to initiate apoptosis and recruit macrophages at sites destined to undergo cell death\textsuperscript{74}. On the contrary, in certain cases senescent cells have been shown to be resistant to apoptotic stimuli\textsuperscript{75,76}. Nonetheless, senescent cells within the developing limb can be detected via senescence associated \(\beta\)-galactosidase (SA-\(\beta\)gal) activity\textsuperscript{74}. It is currently unknown whether BMPs and FGFs regulate limb morphogenesis during development through a senescence dependent mechanism.
1.8 Vascular patterning in interdigit programmed cell death regulation

Recently the role of tissue oxygenation and vascular patterning in limb morphogenesis has been elucidated. During normal development the ID vasculature, which is associated with higher oxygen levels, increases prior to the onset of ID cell death initiation. Eshkar-Oren et al. elegantly demonstrated that a decrease of ID vasculature through the genetic inactivation of Vegf with Prx1-Cre decreases ID cell death and levels of reactive oxygen species (ROS). Conversely, this group further demonstrated that upon Vegf overexpression with Prx1-Cre an overall increase in apoptotic cell death and ROS levels was observed. Finally, it was established that ID PCD and ROS levels are oxygen dependent. Thus, the authors concluded that tissue oxygenation through an increase of the vascular network is necessary during normal development for ROS production and proper ID PCD. However, as the authors pointed out, the decrease in ID PCD of Prx1-Cre; Vegf loss-of-function mutants is also associated with an increase in AER-Fgf8 expression and a decrease in ID Msx2 expression. On the other hand, in the Prx1-Cre; Vegf gain-of-function mutants, an increase in Msx2 and a decrease in AER-Fgf8 is observed upon careful examination of the published figures. Since the authors came to different conclusions from the overall analysis of the Prx1-Cre Vegf gain-of function mutant, further examination is necessary to determine how changes in vascular patterning of the autopod can regulate AER-Fgfs, ID-Bmps, and ROS levels.
1.9 Current model for interdigit programmed cell death regulation and outstanding questions

With the increasing genetic tools and the use of the Cre-lox system more precise questions can be addressed in developmental biology\textsuperscript{12}. Although initial investigations into determining how ID PCD is regulated implicate the direct involvement of BMPs, current studies suggest that BMP signaling to the ID mesenchyme does not play a direct role in ID PCD\textsuperscript{42,43}. In limb cultures with an inhibitor of Smad-dependent BMP signaling, Dorsomorphin, there was no decrease in ID cell death even though a decrease in phosphorylated receptor Smads 1, 5, and 8 was observed\textsuperscript{43,78,79}. In addition to AER-FGF secretion being regulated through BMP signaling to the AER through BMPR1A, the current model of ID PCD regulation suggests an antagonistic relationship between RA metabolism in the proximal ID region and AER-FGF signaling to the underlying mesenchyme (\textbf{Figure 1})\textsuperscript{42,43}. In fact, there are several instances throughout embryonic development, and limb development in particular, during which an antagonistic relationship between RA metabolism and FGF signaling is necessary for proper growth and patterning\textsuperscript{43,80-83}. For example, an opposing gradient of RA and FGF along the anterior – posterior axis of the trunk of the developing embryo provides necessary positional cues for the appropriate location of limb bud initiation along the body axis\textsuperscript{83}. Furthermore, in the case of ID PCD regulation it has been demonstrated that FGF8 decreases the synthesis of RA by inhibiting Raldh2 and increases RA degradation through the upregulation of $Cyp26$\textsuperscript{43}. Conversely, RA inhibits $Fgfr1$ expression and the phosphorylation of ERK1/2, which is necessary for FGF signal transduction\textsuperscript{43}. However,
it is still unclear whether BMP signaling to the ID mesenchyme also directly regulates ID PCD.

1.10 Interdigit tissue as a signaling center for digit formation and identity

At approximately E12.5 the limb bud mesenchyme differentiates into Sox9 positive condensations separated by Sox9 negative mesenchyme$^8$. The mesenchymal condensations proliferate and become digital rays that further develop into mature endochondral bones of phalanges separated by functional joints$^{8,45}$. Although the ID tissue is ultimately removed through the genetically regulated process of PCD, it has been hypothesized that, while present during autopod development the ID is a signaling center to the developing digit that is necessary for proper digit formation and identity specification$^8,41,84$.

In particular, BMP signaling from the ID mesenchyme has been implicated in digit formation and specification$^8,41$. Manipulations of the ID mesenchyme of chick embryos, where each digit has a unique and clear identity based on differing number of phalanges, demonstrated that a BMP signal from the ID posterior to the digital ray specifies digit identity during development$^8$. However, currently, genetic evidence is lacking whether ID BMP signaling affects digit specification during limb development (Figure 1).
Figure 1. Current model of interdigit programmed cell death regulation and outstanding questions.
Figure 1. Current model of interdigit programmed cell death regulation and outstanding questions. Multiple pathways have been implicated in the regulation of interdigit (ID) programmed cell death (PCD). In the current model, PCD is thought to be regulated by an antagonistic relationship between FGF secretion from the apical ectodermal ridge and retinoic acid metabolism in the proximal region of the ID mesenchyme. This schematic demonstrates how FGF signaling acts as a cell survival factor while the retinoic acid pathway activates cell death cascades. Due to the lack of genetic evidence, it is still unknown whether ID BMP signaling can directly regulate ID PCD or whether the ID tissue acts as a signaling center to the developing digit necessary for normal outgrowth and skeletogenesis (question marks).
CHAPTER 2. METHODS
2.1 Ethics statement

Mice were treated in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Academies Press; 8th edition). The protocol was approved by the Animal Care and Usage Committee of NCI – Frederick (NIH) (Animal Study Proposal: 11 – 069).

2.2 Tables of genetic crosses used within this thesis

<table>
<thead>
<tr>
<th>Experimental Cross</th>
<th>Control Genotype (Frequency)</th>
<th>Experimental Genotype (Frequency)</th>
<th>(1)</th>
<th>(1)</th>
<th>(1)</th>
<th>(12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RosamTmG/0;Bmp2flox/</td>
<td>NA</td>
<td>RosamTmG/0;Bmp2flox/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>RosamTmG/wt</td>
<td>Osr1%CreTg/0;RosaLacZ/wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Osr1%CreTg/0;Bmp1Bmp2Bmp7flox/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RosamTmG/wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bmp1Bmp2Bmp7flox/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LacZ/wt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*Animals also contain Bmp1Bmp2Bmp7flox/ and/or Bmp1Bmp2Bmp7null/.
**Animals also contain Bmp1Bmp2Bmp7flox/ and/or Bmp1Bmp2Bmp7null/.
***Animals also contain Bmp1Bmp2Bmp7flox/ and/or Bmp1Bmp2Bmp7null/.
Table 2. Chapter 4 Genetic Crosses

<table>
<thead>
<tr>
<th>Experimental Cross</th>
<th>Experimental Genotype (Frequency)</th>
<th>Control Genotype (Frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Bmp^{2flox/flox}$ ** X $Bmp^{2flox/flox}$</td>
<td>Osr1-CreTg0; $Bmp^{2flox/flox}$ (1/2)</td>
<td>$Bmp^{2flox/flox}$ * (1/2)</td>
</tr>
<tr>
<td>$Bmp^{4flox/flox}$ ** X $Osr1-CreTg0; Bmp^{4null/wt}$</td>
<td>Osr1-CreTg0; * $Bmp^{2flox/flox}$ (1/4)</td>
<td>$Bmp^{4flox/null}$ * (1/4)</td>
</tr>
<tr>
<td>$Bmp^{7flox/flox}$ ** X $Osr1-CreTg0; Bmp^{7flox/flox}$</td>
<td>Osr1-CreTg0; * $Bmp^{2flox/flox}$ (1/2)</td>
<td>$Bmp^{7flox/flox}$ * (1/2)</td>
</tr>
<tr>
<td>$Bmp^{2flox/flox}$ **; $Bmp^{4flox/flox}$ X $Osr1-CreTg0; Bmp^{2flox/flox}$</td>
<td>Osr1-CreTg0; * $Bmp^{2flox/flox}$ (1/2)</td>
<td>$Bmp^{2flox/flox}$ * (1/2)</td>
</tr>
<tr>
<td>$Bmp^{4flox/flox}$ **; $Bmp^{7flox/flox}$ X $Osr1-CreTg0; Bmp^{4flox/flox}$</td>
<td>Osr1-CreTg0; * $Bmp^{2flox/flox}$ (1/2)</td>
<td>$Bmp^{4flox/flox}$ * (1/2)</td>
</tr>
<tr>
<td>$Bmp^{2flox/wt}$; $Bmp^{7flox/wt}$ X $Osr1-CreTg0; Bmp^{2flox/wt}$</td>
<td>Osr1-CreTg0; * $Bmp^{2flox/wt}$ (1/16)</td>
<td>Osr1-CreTg0; $Bmp^{7flox/wt}$ * (1/16)</td>
</tr>
<tr>
<td>$Bmp^{2flox/flox}$ **; $Bmp^{4flox/flox}$; $Bmp^{7flox/flox}$ X $Osr1-CreTg0; Bmp^{4flox/flox}$; $Bmp^{7flox/flox}$</td>
<td>Osr1-CreTg0; * $Bmp^{2flox/flox}$ (1/2)</td>
<td>$Bmp^{2flox/flox}$ * (1/2)</td>
</tr>
</tbody>
</table>

* Animals also contain Rosa$mTmG$/wt
** Animals also contain Rosa$mTmG$
Table 3. Chapter 5 Genetic Crosses

<table>
<thead>
<tr>
<th>Experimental Cross</th>
<th>Experimental Genotype (Frequency)</th>
<th>Control Genotype (Frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmpr1a&lt;sup&gt;flox/flox&lt;/sup&gt; ** X Osr1-Cre&lt;sup&gt;Tg/Tg;&lt;/sup&gt; Bmpr1a&lt;sup&gt;null/wt&lt;/sup&gt;</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Bmpr1a&lt;sup&gt;flox/null&lt;/sup&gt;&lt;/sup&gt; (1/2)</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Bmpr1a&lt;sup&gt;flox/wt&lt;/sup&gt;&lt;/sup&gt; (1/2)</td>
</tr>
<tr>
<td>Bmpr1a&lt;sup&gt;flox/flox&lt;/sup&gt;, Bmpr1b&lt;sup&gt;null/wt&lt;/sup&gt; ** X Osr1-Cre&lt;sup&gt;Tg/Tg;&lt;/sup&gt; Bmpr1a&lt;sup&gt;null/wt&lt;/sup&gt;, Bmpr1b&lt;sup&gt;null/wt&lt;/sup&gt;</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Bmpr1a&lt;sup&gt;flox/null&lt;/sup&gt;&lt;/sup&gt; (1/8)</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Bmpr1a&lt;sup&gt;flox/wt&lt;/sup&gt;&lt;/sup&gt; (1/8)</td>
</tr>
<tr>
<td>Smad4&lt;sup&gt;flox/flox&lt;/sup&gt; X Osr1-Cre&lt;sup&gt;Tg/Tg;&lt;/sup&gt; Smad4&lt;sup&gt;null/wt&lt;/sup&gt;</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Smad4&lt;sup&gt;flox/null&lt;/sup&gt;&lt;/sup&gt; (1/2)</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Smad4&lt;sup&gt;flox/wt&lt;/sup&gt;&lt;/sup&gt; (1/2)</td>
</tr>
<tr>
<td>Bmpr1a&lt;sup&gt;flox/flox&lt;/sup&gt; ** X Osr1-Cre&lt;sup&gt;Tg/0;&lt;/sup&gt; Msx2-Cre&lt;sup&gt;Tg/0;&lt;/sup&gt; Bmpr1a&lt;sup&gt;null/wt&lt;/sup&gt;</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Msx2-Cre&lt;sup&gt;Tg/0;&lt;/sup&gt; Bmpr1a&lt;sup&gt;flox/null&lt;/sup&gt;&lt;/sup&gt; (1/4)</td>
<td>Osr1-Cre&lt;sup&gt;Tg/0; Msx2-Cre&lt;sup&gt;Tg/0;&lt;/sup&gt; Bmpr1a&lt;sup&gt;flox/wt&lt;/sup&gt;&lt;/sup&gt; (1/4)</td>
</tr>
</tbody>
</table>

* Animals also contain Rosa<sup>mTmG/wt</sup>
** Animals also contain Rosa<sup>mTmG/imTmG</sup>
### Table 4. Chapter 6 Genetic Crosses

<table>
<thead>
<tr>
<th>Experimental Cross</th>
<th>Experimental Genotype (Frequency)</th>
<th>Control Genotype (Frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp2\text{flox/flox}, ** X Bmp4\text{flox/flox}, Bmp7\text{flox/flox}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp2\text{flox/flox}, Bmp4\text{flox/flox}, Bmp7\text{flox/flox}}</td>
<td>Bmp2\text{flox/flox}, * Bmp4\text{flox/flox}, Bmp7\text{flox/flox} (1/2)</td>
</tr>
<tr>
<td>Bmpr1a\text{flox/flox}, ** X Bmpr1b\text{null/wt}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}}; Bmpr1b\text{null/wt}}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}}; Bmpr1b\text{null/wt}} (1/8)</td>
</tr>
<tr>
<td>Bmpr1b\text{null/wt} X Bak\text{null/wt}</td>
<td>Bak\text{null/null, Bax\text{null/wt}}</td>
<td>Bak\text{null/null} (1/4)</td>
</tr>
<tr>
<td>Bmpr1b\text{null/wt} X Bak\text{null/wt}</td>
<td>Bak\text{null/null, Bax\text{null/wt}}</td>
<td>Bak\text{null/null} (1/8)</td>
</tr>
<tr>
<td>Bmpr1a\text{flox/flox}, Bmpr1b\text{null/wt} X Bak\text{null/wt}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}, Bmpr1b\text{null/wt}, Noggin\text{LacZ/0']]}}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}, Bmpr1b\text{null/wt}, Noggin\text{LacZ/0}]}} (1/16)</td>
</tr>
<tr>
<td>Bmpr1b\text{null/wt} X Bak\text{null/wt}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}, Bmpr1b\text{null/wt}, Noggin\text{LacZ/0}]}}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}, Bmpr1b\text{null/wt}, Noggin\text{LacZ/0}]}} (1/16)</td>
</tr>
<tr>
<td>Bmpr1a\text{flox/flox}, Bmpr1b\text{null/wt} X Bak\text{null/wt}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}, Bmpr1b\text{null/wt}, Noggin\text{LacZ/0}]}}</td>
<td>Osr1\text{Cre\textsuperscript{Tg/0}; Bmp1\text{a\text{null/wt}, Bmpr1a\text{null/wt}, Bmpr1b\text{null/wt}, Noggin\text{LacZ/0}]}} (1/16)</td>
</tr>
</tbody>
</table>

* Animals also contain Rosa\text{mTmG}\text{wt}

** Animals also contain Rosa\text{mTmG}\text{tmG}
2.3 Mouse line maintenance and genotyping

Mice were kept on a mixed background consisting of NIH/Swiss/129SvEv/C57BL/6. Genotyping was performed as previously reported for the following alleles or transgenes: Osr1-Cre\textsuperscript{86}, Rosa\textsuperscript{87}, Rosa\textsuperscript{88}, Bmpr1a null\textsuperscript{89}, Bmpr1a flox and wt\textsuperscript{90}, Bmpr1b null and wt\textsuperscript{91}, Bmp2 null, flox and wt\textsuperscript{70,92}, Bmp4 null, flox, and wt\textsuperscript{70,93,94}, Bmp7 null, flox and wt\textsuperscript{95,96}, Smad4 null, flox and wt\textsuperscript{97}, Bak null and wt\textsuperscript{98}, Bax null and wt\textsuperscript{98}, Noggin\textsuperscript{99}. A list of relevant primer set sequences for the corresponding polymerase chain reaction (PCR) is provided in Table 1. In some instances, EGFP fluorescence was used to determine the presence of the Osr1-Cre transgene instead of a PCR genotyping reaction.
Table 5. List of relevant PCR reactions with corresponding primer sequences.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Primer set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osr1-Ctc</td>
<td>Osr1-Ctc F: GCT CCA GGT TTT TCT GAA TGT GGA</td>
</tr>
<tr>
<td></td>
<td>Osr1-Cre R: TTC AGG TTC TGC GGG GAA CCC</td>
</tr>
<tr>
<td>Rosa mTmG</td>
<td>mTmG F1: CTC TGC TGC TGC TGC GGT TCT TCT</td>
</tr>
<tr>
<td></td>
<td>mTmG F2: CGA GGC GGA TCA CAA GCA GAT A</td>
</tr>
<tr>
<td></td>
<td>mTmG R: TCA ATG GCC GGG GGT CGT T</td>
</tr>
<tr>
<td>Rosa LacZ</td>
<td>R26R F1: GGA GGC GGA GAA ATG GAT ATG</td>
</tr>
<tr>
<td></td>
<td>R26R F2: GCC AAG ATG TTT TCC TCA ACC</td>
</tr>
<tr>
<td></td>
<td>R26R R: AAA GTC GCT CTG AGT GGT TAT</td>
</tr>
<tr>
<td>Bmp4 1a null/wt</td>
<td>Bmp4 1a wtR: TGG ACA CAC AAT GGC TGA GC</td>
</tr>
<tr>
<td></td>
<td>Bmp4 1a wtF: GGT ATG CAC CAC TAT GCC TG</td>
</tr>
<tr>
<td></td>
<td>Bmp4 1a delF: CAC TTT TGT GAT TAC CAC</td>
</tr>
<tr>
<td>Bmp4 1a flox/wt</td>
<td>Bmp4 1a floxF: CAA TTT GTC TCA TGC TCT AGC AGG GAA</td>
</tr>
<tr>
<td></td>
<td>Bmp4 1a floxR: TCC CTG TGT CAG GGT ATC TCC AACC</td>
</tr>
<tr>
<td>Bmp4 1b wt</td>
<td>Bmp4 1b wtR: TGG TGA GTG GTT ACA ACA AGG TCA GCA</td>
</tr>
<tr>
<td></td>
<td>Bmp4 1b R: ATG CTG GGC ATG CCG TG</td>
</tr>
<tr>
<td>Bmp4 1b null</td>
<td>Bmp4 1b null F: TGG TGA GTG GTT ACA ACA AGG TCA GCA</td>
</tr>
<tr>
<td></td>
<td>Bmp4 1b null R: GGC AGA CAT GTG AIC TCT AGG</td>
</tr>
<tr>
<td>Bmp4 2 null</td>
<td>Bmp4 2 F: GGC AGA CAT GTG AIC TCT AGG</td>
</tr>
<tr>
<td></td>
<td>Bmp4 2 null R: TGC CAT CAT CAC TCC TCT AC</td>
</tr>
<tr>
<td>Bmp4 null, flox/wt</td>
<td>Bmp4 null F: GCC AGA CAT GTG AIC TCT AGG</td>
</tr>
<tr>
<td></td>
<td>Bmp4 null R: GGC AGA CAT GTG AIC TCT AGG</td>
</tr>
<tr>
<td>Bmp4 flox/wt</td>
<td>Bmp4 floxF: GCC CAA TTT CAA CAA CTT CCA GC</td>
</tr>
<tr>
<td></td>
<td>Bmp4 floxR: CAC AAC AGG TGA GCA GAC GCA</td>
</tr>
<tr>
<td>Bmp7 null</td>
<td>Bmp7 7 F: AAG CCA GCC TCG CTG ATT G</td>
</tr>
<tr>
<td></td>
<td>Bmp7 null R: TTT AGC CCC TCA GAC AGT CAC</td>
</tr>
<tr>
<td>Bmp7 flox/wt</td>
<td>Bmp7 7 F: AAG CCA GCC TCG CTG ATT G</td>
</tr>
<tr>
<td></td>
<td>Bmp7 floxR: GCC TGA GGC TCA GAG GCT ATG</td>
</tr>
<tr>
<td>Smad 4 null</td>
<td>Smad 4 F: GCC CAA ACC GTC ACC TTC AG</td>
</tr>
<tr>
<td></td>
<td>Smad 4 null: GCC CAA ACC GTC ACC TTC AG</td>
</tr>
<tr>
<td>Bmp7 flox/wt</td>
<td>Smad 4 F: GCC CAA ACC GTC ACC TTC AG</td>
</tr>
<tr>
<td></td>
<td>Smad 4 floxR: AAG AGC CAC AGG TCA AGC AG</td>
</tr>
<tr>
<td>Bak null</td>
<td>Bak 2: GTC TAG CAG GCC TTC GAA CGG</td>
</tr>
<tr>
<td></td>
<td>Bak 1: GCC TCT TCA CAC CTT ACA TCA G</td>
</tr>
<tr>
<td>Bak wt</td>
<td>Bak 2: GTC TAG CAG GCC TTC GAA CGG</td>
</tr>
<tr>
<td></td>
<td>Bak 3: GCA GGC CAT CGC CTT CTA TC</td>
</tr>
<tr>
<td>Dax null</td>
<td>Dax null F: GAA CCC TAG GAC CCC TCC G</td>
</tr>
<tr>
<td></td>
<td>Dax null R: CAA CTC CTA CCG CAA GTC CTC G</td>
</tr>
<tr>
<td>Dax wt</td>
<td>Dax wt F: TGC CGA ACT GGG CAC TGT TG</td>
</tr>
<tr>
<td></td>
<td>Dax wt R: GTC TGT GGG AAT GGT GAC TG</td>
</tr>
<tr>
<td>Noggin-LacZ</td>
<td>Noggin F: GCA TGG AGC GCT GCC CCA GC</td>
</tr>
<tr>
<td></td>
<td>Noggin R: GAA CAG CTA GCA CAG CAG CAG</td>
</tr>
<tr>
<td></td>
<td>Noggin R2: AAG GGC GAT CGG TGC GGG CC</td>
</tr>
</tbody>
</table>
2.4 LysoTracker Red assay for cell death detection

Total cell death in embryos was detected using LysoTracker® Red (Molecular Probes L-7528) as previously described\textsuperscript{100}. Embryos were dissected in 37°C Hank’s balanced salt solution (HBSS) and then transferred either to a 15mL conical tube or a 24 well plate containing a solution of 5 µl of LysoTracker Red per mL of HBSS. The embryos were incubated in this solution for 30 min in a 37°C incubator under normal atmospheric conditions while rocking. Next, embryos were rinsed briefly with HBSS and fixed with 4% paraformaldehyde in PBS (PFA), while rocking, either at room temperature for 30 minutes or at 4°C overnight. Subsequently, embryos were dehydrated stepwise (PBS:Methanol series) to 100% methanol at room temperature. Embryos were stored in 100% Methanol until time for imaging, up to a couple of months. Prior to imaging, embryos were rehydrated stepwise to PBS at room temperature and either imaged in PBS or moved to a 50% glycerol:PBS solution.

2.5 X-gal staining for β-galactosidase activity and sectioning

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining for β-galactosidase activity was performed on embryos as previously described\textsuperscript{101} either in whole mounts or on 20 µm thick frozen sections. Briefly, for whole mount staining, embryos were fixed with 4% PFA for 30 min at room temperature. Then, after a couple of brief washes with PBS, embryos were incubated overnight rocking at 37°C in standard X-gal stain\textsuperscript{101}. Embryos were either stored in 4% PFA for whole mount imaging or embedded in plastic blocks for sectioning. For plastic block embedding, after staining, embryos were fixed overnight in 4% PFA and then dehydrated stepwise into 100% methanol. Then, embryos
were embedded using the JB – 4 embedding kit as per manufacturer’s instructions (Polysciences Inc. Catalog# 00226). Five µm sections were counterstained with neutral red. For frozen sections, P0.5 limbs were fresh frozen using O.C.T. compound (VWR Catalog# 25608-930). 20 µm sections were fixed for 10 minutes at room temperature with PFA:glutaraldehyde and then rinsed in PBS. Sections were incubated overnight with filtered, standard X-gal stain. Slides were briefly washed with PBS, then water, and counterstained for approximately one minute with 0.1% Neutral red.

2.6 Whole mount in situ hybridization

Whole mount in situ hybridization was performed as previously described using digoxigenin-labeled riboprobes and alkaline phosphatase-conjugated anti-digoxigenin antibody, followed by the chromogenic substrate BM Purple (Roche)\textsuperscript{101}. In all cases, experimental and control embryos were processed in the same tube throughout the experiment.

2.7 Whole mount immunolabeling

Whole mount immunolabeling was performed as previously described with some minor variations\textsuperscript{102}. Embryos were fixed overnight with 4% PFA in PBS at 4\textdegree{}C. Then embryos were dehydrated stepwise to 100% methanol and stored at -20\textdegree{}C at least for one overnight. For whole mount immunolabeling, embryos were transferred to freshly prepared methanol/DMSO/H\textsubscript{2}O\textsubscript{2} as described\textsuperscript{102} for up to 10 hours rocking at room temperature. The following primary antibodies from cell signaling technologies were used at a concentration of 1:100 – cleaved Caspase 3 (Asp175) #9661 and
phosphorylated Smad 1 (Ser463) and 5 (Ser465) #9516. PECAM-1 (platelet endothelial cell adhesion molecule) (CD31) was obtained from BD Pharmingen (PMG550274) and used at a concentration of 1:25. In the case of cleaved Caspase 3 and phosphorylated Smad 1 and 5, a goat anti rabbit secondary was used at a concentration of 1:500 and visualized using DAB/NiCl (3,3’-diaminobenzidine tetrahydrochloride / NiCl2) substrates as previously published102. For PECAM detection, a biotin-SP-conjugated AffiniPure donkey anti-rat Ig (Jackson Lab. 712-065-153) was used at a 1:100 dilution followed by a one hour room temperature incubation with Alexa fluor 488-streptavidin (Jackson Lab. 016-540-084) at a 1:100 dilution. Confocal microscopy was used to detect fluorescent staining. In all cases, experimental and control embryos were processed in the same tube throughout the experiment.

2.8 Senescence associated β-galactosidase assay

Senescence associated β-galactosidase activity was performed as previously described except at a pH of 5.7574,103.

2.9 Reactive oxygen species detection

Reactive oxygen species were detected using Dihydroethidium (DHE) (Life Technologies D-1168) per manufacturer instructions and as previously described77.

2.10 Confocal microscopy

Confocal imaging was performed using a LSM780 laser scanning confocal microscope (CarlZeiss). Z-stacks were acquired at 0.5 airy unit (AU) increments. Images
were processed offline utilizing Fiji (version of ImageJ) software (National Institutes of Health).

2.11 Skeletal preparations

Skeletal preparations were performed using standard protocols as previously described\(^{104}\).

2.12 Planar X-ray imaging

Planar X-Ray analysis of limbs of euthanized adult mice was performed through the small animal imaging program (SAIP) at NCI – Frederick.

2.13 Mouse interdigit RNA isolation

Interdigit tissue was collected in ice cold PBS, quick frozen using liquid nitrogen, and stored at \(-80\degree\text{C}\) until samples were genotyped. To obtain high purity total RNA for microarray experiments, RNA was first isolated using TRIzol reagent (Ambion) and a protocol modified from Invitrogen (Cat# 155596-026). 50 µl of TRIzol was added per ID and left at room temperature (RT) for a couple of minutes. Next, the ID was vortexed for \(~30\) seconds and the left and right IDs from two embryos of the same genotype were pooled (200 µl total). Samples were left for a couple of minutes at RT and vortexed for another 30 seconds. Next, 40 µl of Chloroform per 200 µl of TRIzol were added and tubes were shaken vigorously by hand for 15 seconds followed by a 3 minute incubation at RT. After a 15 minute 12,000 x g centrifuge spin at 4\degree\text{C}, the upper aqueous layer was transferred to a new 1.5mL eppendorf tube. 100 µl of isopropanol and 1 µl of glycogen
were added to this new tube, mixed by flicking, and incubated at RT for 15 minutes. After a 15 minute 12,000 x g centrifuge spin at 4°C, the RNA pellet was washed with 200 µl of 75% ethanol and centrifuged again at 12,000 x g for 15 minutes at 4°C. The liquid was removed; the RNA pellet air dried for no more than 5 minutes, and dissolved in 43 µl AccuGENE water (Lonza Cat# 51200). To remove DNA contamination, 5 µl of 10X TurboDNAse buffer and 2 µl of TurboDNAse enzyme (Life Technologies AM2238) were added and incubated for 20 minutes at 37°C. Volume was brought up to 150 µl and a Phenol:Chloroform: isoamyl alcohol purification was done with glycogen as a carrier and 3M pH5.2 sodium acetate. RNA was resuspended in 10 µl water and approximately 1.5 µl was run on an Agilent Bioanalyzer using the PICO kit to determine concentrations and quality. The quality control cut-off was a RIN score of ≥ 7.9.

2.14 Microarray expression profiling

Microarray expression profiling was performed by the Laboratory of Molecular Technology, the CSAS, and the Advanced Technology Program at Frederick, Maryland. Briefly, approximately 10ng of total RNA was utilized as starting material for cDNA synthesis and GeneChip Mouse Genome 430 2.0 arrays were used for hybridization (Affymetrix, Santa Clara, CA). Both forelimb IDs from six embryos were used for each array and array hybridizations were performed in quadruplicate per genotype. Subsequent analyses were performed on probe-intensity level data (CEL files) using GeneSpringGX software.
2.15 Measurements and statistics

For all analyses, at least three specimens were examined unless otherwise specified. For measurements of skeletal elements, length was measured in pixels within Adobe Photoshop CS4 (Version 11.0) using the measuring tool. The length of each skeletal element was determined by calculating the mean of three independent measurements. Significance was determined using an unpaired two-tailed t-test.
CHAPTER 3. EVALUATION OF PROGRAMMED CELL DEATH INITIATION
AND PROGRESSION DURING NORMAL MOUSE LIMB DEVELOPMENT
AND RELATIVE TO OSRI-CRE ACTIVITY
Limb development requires the dynamic coordination of signaling from multiple tissues. It is especially important to characterize how the timing of the dynamic changes of AER-Fgf8 down-regulation and ID PCD coincide with Osr1-Cre expression. This allows us to determine the scope of questions we can address with our genetic tools and gain a more precise understanding of how information from different signaling pathways is coordinated in ID PCD regulation during normal mouse limb development.

3.1 Characterizing programmed cell death initiation and progression during limb development via LysoTracker Red

To gain a better understanding of the spatio-temporal progression and regulation of PCD, LysoTracker Red was utilized to mark all dying cells of the mouse hindlimb at several developmental stages. In the mixed genetic background, ID PCD has not initiated at around E12.5 in the developing hindlimbs (Figure 2A). At approximately E13.5, it is evident that PCD is first initiated in the outer IDs (ID1 and ID4) while remaining relatively low or absent in the middle IDs of the autopod (ID2 and ID3) (Figure 2B). Furthermore, if ID1 is compared between panels B and C of Figure 1, it appears that the process of PCD is initiated at the distal part within an ID and extends proximally throughout development. Based on our observations, in our genetic background, PCD is fully initiated through the whole ID region around E13.75 (Figure 2 C and D).

3.2 Characterizing Osr1-Cre activity and timing relative to mouse limb development

The odd skipped related (Osr) genes are mammalian zinc finger transcription factors\textsuperscript{105}. The Osr1-Cre transgenic mouse was previously developed using bacterial
artificial chromosome (BAC) transgenesis\textsuperscript{86}. Although the authors originally attributed Osr1-Cre expression to a position effect\textsuperscript{86}, later groups observed an endogenous Osr1 expression domain in the proximal ID region of mouse developing limbs\textsuperscript{105}, a region where Osr1-Cre mediated recombination is also detected. To detect Cre mediated recombination the \textit{Rosa} \textsuperscript{mTmG} reporter was utilized\textsuperscript{87}. In the presence of CRE protein the gene encoding a myristoylated Tomato fluorophore is excised and the gene encoding a myristoylated \textit{eGFP} is expressed\textsuperscript{87}. Thus, the spatiotemporal pattern of Cre-mediated recombination can be detected via eGFP fluorescence. In the hindlimb, Osr1-Cre activity initiates at the junction of autopod and zeugopod (\textbf{Figure 2E}). As development progresses, Cre activity in this domain becomes stronger and is additionally observed throughout the ID mesenchyme (\textbf{Figure 2F-H}). An anterior to posterior gradient in EGFP intensity is observed both in timing of activation and total fluorescence. While EGFP fluorescence intensity, and thus Cre activity, is strongest in ID1, it remains minimal in ID4 (\textbf{Figure 2H}).

To confirm that Osr1-Cre activity is limited to the ID mesenchyme, the Gt(ROSA)26Sortm1Sor (R26R) reporter mouse line was used\textsuperscript{88}. With this tool Osr1-Cre activity can be assayed via the Cre-mediated activation of the near-ubiquitously transcribed \textit{LacZ} gene that encodes beta-galactosidase (\textit{β}gal). \textit{β}gal activity can then be detected by X-gal histochemistry. Cre activity was examined in sections at both E13.5 and postnatal day (P) 0.5 (\textbf{Figure 3}). At E13.5, X-gal was detected in whole mount hindlimbs (\textbf{Figure 3}), which were embedded in plastic blocks and sectioned to confirm that X-gal positive cells were restricted to the ID mesenchyme (\textbf{Figure 3A'-A''}). At P0, \textit{β}gal activity was detected in 20 µm frozen sections (\textbf{Figure 3B-C}). At this stage, some
X-gal positive cells can be detected around the periphery of digit one (Figure 3B'). However, the number of blue X-gal cells in the digits decrease in the posterior digits two through four (Figure 3C). This is consistent with the observed anterior to posterior gradient in Osr1-Cre activity.

Another important observation is the apparent variability of Cre activity in the developing autopods between littermates (Figure 4). Consequently, the Rosa\textsuperscript{mTmG} reporter line was used for all experimental crosses and only embryos with strong Osr1-Cre recombination were used for further analysis (Figure 4D) but not those with low or heterogeneous Osr1-Cre activity (Figure 4E-F).

### 3.3 Changes in AER-Fgf8 mRNA expression

The apical ectodermal ridge is necessary for proper limb bud outgrowth, development, and patterning\textsuperscript{25-27,29,30,106}. In particular, FGF secretion from the AER to the underlying mesenchyme is required during these processes\textsuperscript{25-27,29,30,106}. AER-Fgf8 expression is regulated through BMP signaling within the AER during autopod development\textsuperscript{42}. For example, the tissue-specific inactivation of Bmpr1a in the AER with Msx2-Cre results in an up-regulation of AER-Fgf4 and Fgf8 expression, a decrease in ID cell death, and a syndactylous phenotype\textsuperscript{42}. Similarly, a decrease in ID cell death and a syndactylous phenotype due to an increase in AER-Fgf expression above the ID mesenchyme is observed with other tissue-specific inactivations of Bmp related genes\textsuperscript{26,70,72,107}. Since it has been established that AER-FGF signaling to the underlying mesenchyme regulates ID PCD, it is important to characterize how the spatiotemporal regulation of Fgf8 downregulation correlates with the initiation of ID PCD and the timing
of Osr1-Cre activity. To accomplish this, AER-Fgf8 expression was examined in hindlimbs for which total cell death and Osr1-Cre activity were determined. At approximately E12.75, prior to ID cell death and detectable Osr1-Cre activity in the ID mesenchyme, Fgf8 is expressed throughout the AER (Figure 2I). By ~ E13.5, AER-Fgf8 expression is mostly absent in the mesenchyme above ID1 and ID4 and has begun to be downregulated above ID2 and ID3 (Figure 2J). As autopod development progresses, Fgf8 expression is further down regulated and becomes absent in the AER overlying the ID mesenchyme while ID regression can be detected (Figure 2K-L). It appears that the extent and magnitude of total cell death is the greatest in ID regions above which AER-Fgf8 expression is absent (compare Figure 2 B and J).

3.4 Characterizing interdigit BMP activity via changes in Msx2 mRNA expression during programmed cell death

There is evidence that the role of BMPs in the regulation of PCD is through the activation of the Msx homeobox genes\textsuperscript{56,108-111}. Therefore, Msx2 expression is often used as a readout of BMP activity in the ID mesenchyme\textsuperscript{42,43}. Mutant mice with inactivated Msx1 and Msx2 show a decrease in cell death and retain their ID mesenchyme indicating that the Msx genes are involved in the regulation of PCD\textsuperscript{48,112}. During limb development Msx2 expression is detected throughout the whole ID mesenchyme and sub-AER regions prior to cell death initiation and Osr1-Cre expression (Figure 2M). As autopod development proceeds and the digit condensations further develop, Msx2 expression becomes more restricted to the ID (Figure 2N-P). After approximately E14.0, Msx2 expression begins to be downregulated well after ID PCD has been initiated.
Figure 2. Characterization of programmed cell death initiation and progression during normal mouse limb development and relative to Osr1-Cre activity.

Table:

<table>
<thead>
<tr>
<th></th>
<th>E12.75</th>
<th>E13.5</th>
<th>E13.75</th>
<th>E14.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Death</td>
<td><img src="A" alt="Image" /></td>
<td><img src="B" alt="Image" /></td>
<td><img src="C" alt="Image" /></td>
<td><img src="D" alt="Image" /></td>
</tr>
<tr>
<td>EGFP</td>
<td><img src="E" alt="Image" /></td>
<td><img src="F" alt="Image" /></td>
<td><img src="G" alt="Image" /></td>
<td><img src="H" alt="Image" /></td>
</tr>
<tr>
<td>Fgf8</td>
<td><img src="I" alt="Image" /></td>
<td><img src="J" alt="Image" /></td>
<td><img src="K" alt="Image" /></td>
<td><img src="L" alt="Image" /></td>
</tr>
<tr>
<td>Msx2</td>
<td><img src="M" alt="Image" /></td>
<td><img src="N" alt="Image" /></td>
<td><img src="O" alt="Image" /></td>
<td><img src="P" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 2. Characterization of programmed cell death initiation and progression during normal mouse limb development and relative to Osr1-Cre activity.

*Rosa^mTmG/mTmG* homozygous females were mated to Osr1-Cre homozygous males. Immediately following dissection at ~E12.75 - ~E14.0, EGFP fluorescence was examined in both left and right hindlimbs to assess extent of Osr1-cre activity (E-H). The LysoTracker Red assay was used to examine total cell death in the developing limb (A-D). Individual embryos were split in half along the anterior – posterior axis and RNA *in situ* hybridization was performed to detect *Fgf8* (I-L) expression on the right limb and *Msx2* (M-P) expression on the left limb. Therefore, for each developmental time point LysoTracker Red, EGFP, and *Fgf8* expression is examined on the same limb (e.g. B,F,J). Since the left hindlimbs were used to examine *Msx2* expression, the images were flipped horizontally so that anterior would remain to the left (M-P). Note that ID cell death is first initiated in ID1 and ID4 (B, white arrows) and a few hours later PCD is initiated in ID2 and ID3 (C, white arrows). Comparing panels A and I, it can be observed that there is no ID cell death while *Fgf8* expression remains above the ID mesenchyme (yellow arrows). At a later developmental time point, (comparing panels B and J) in ID1, where there is extensive cell death, *Fgf8* expression is not detected in the apical ectodermal ridge overlying the ID mesenchyme (yellow arrow). *Msx2*, a BMP responsive gene, is expressed throughout the ID mesenchyme before PCD is initiated and through ~E14.0 (M-P). ID, interdigit.
Figure 3. Characterization of Osr1-Cre activity in the developing mouse autopod.

To closely examine the pattern of Osr1-Cre recombination, embryos at E13.5 (A-A’’) and neonates at P0 (B-C) carrying the Osr1-Cre transgene were assayed for R26R activation via staining for β-galactosidase activity (blue). At E13.5, upon detection of Osr1-Cre activity in whole mount (A), the hindlimb was sectioned laterally (A’ and A’’). It is evident that Osr1-Cre activity is restricted to the ID mesenchyme at E13.5. At P0, β-galactosidase activity was assayed on 20µm frozen sections. Blue cells can be observed in the periphery of the developing digit one (B-B’) while β-galactosidase positive cells are mostly absent in digits two through four (C). Relevant genotypes are shown, but may also carry Bmpr1a<sup>null/wt</sup> and/or Bmpr1b<sup>null/wt</sup>. D1, digit 1; D2, digit 2; D3, digit 3; D4, digit 4.
Figure 4. **Variability of Osr1-Cre deletion efficiency.** Although the pattern of activity is consistent, differences in Osr1-Cre activity can be detected when using established Cre-reporter strains such as $\text{Rosa}^{\text{LacZ/LacZ}}$ (A-C) and $\text{Rosa}^{\text{mTmG/mTmG}}$ (D-F). Throughout this work, most experimental crosses carry the $\text{Rosa}^{\text{mTmG/mTmG}}$ reporter so that embryos with low Cre activity can be discarded (E and F) and only embryos with high deletion efficiency can be used for further analysis (D). Relevant genotypes are shown, but also carry one or all of the following: $\text{Bmp2}^{\text{flox/wt}}$, $\text{Bmp4}^{\text{flox/wt}}$, $\text{Bmp7}^{\text{flox/wt}}$ (A-C) or may carry one or both of: $\text{Bmpr1a}^{\text{null/wt}}$, $\text{Bmpr1b}^{\text{null/wt}}$ (D-F).
3.5 Summary

A careful examination of Osr1-Cre activity showed that it is mostly restricted to the ID limb mesenchyme with an anterior to posterior gradient, strongest in the anterior. A notable difference in levels of Cre activity was sometimes observed within a litter or between littermates. It appears that the timing of AER-Fgf8 down-regulation and ID PCD initiation is linked, as the AER overlying areas of robust ID cell death lacks Fgf8 expression. ID PCD is initiated and is most extensive in ID1 and ID4 and then progresses to ID2 and ID3. Within ID1, Osr1-Cre recombination and ID PCD initiation occur at similar times. However, in ID2 and ID3, Osr1-Cre activity is observed prior to robust ID cell death. There is extensive ID Msx2 expression at least 12 hours prior to ID PCD initiation, which remains throughout ID PCD progression.

3.6 Discussion

Previously it was shown that BMP signaling could regulate ID PCD indirectly through FGF expression in the AER42. However, it is still unknown whether ID BMP signaling can directly regulate ID PCD. With the availability of the ID mesenchymal tissue-specific Osr1-Cre transgenic mouse line, it is possible to genetically manipulate different aspects of the BMP signaling cascade specifically within the ID mesenchyme86. Because limb development is such a dynamic process that integrates multiple complex signaling cascades, it is important to establish how AER-Fgf8 expression and Msx2 expression are changing relative to the initiation and progression of ID PCD as well as activation of the Osr1-Cre transgene. Through this analysis, I determined that at the developmental time point when Cre activity is observed throughout the ID mesenchyme,
downregulation of $Fgf8$ expression has begun in the AER overlying the ID mesenchyme. Thus, the Osr1-Cre allows tissue-specific manipulation of BMP signaling to test whether ID ligand signaling affects AER-$Fgf8$ expression. Furthermore, due to the anterior-posterior gradient of Osr1-Cre activity, any phenotypes resulting from a change in ID BMP activity might be greatest in ID1 and least in ID4, possibly allowing for the use of ID4 as an internal control. Similarly, a difference in phenotype could be observed between the middle two IDs and ID1, since Osr1-Cre is active longer in the middle IDs before PCD is initiated. Finally, due to variability in Osr1-Cre activity that could result in variability in observed phenotypes, it was established that experimental embryos must be pre-screened using the $Rosa^{mTmG}$ reporter before conducting further analyses.
CHAPTER 4. INTERDIGIT BMP 7 LIGAND SECRETION IS NECESSARY FOR
PROGRAMMED CELL DEATH DURING NORMAL DEVELOPMENT
One approach to determine whether ID BMP signaling regulates PCD during normal development is through the ID tissue-specific inactivation of genes encoding BMP ligands expressed around the time of PCD initiation. In addition, since it has been shown that BMP signaling to the AER through BMPR1A regulates AER-Fgf expression and PCD\textsuperscript{42}, inactivating BMP ligands from ID tissue may also address whether secretion of BMP ligands from the ID mesenchyme could signal to the AER.

4.1 Interdigit Bmp 2, 4, 7 expression domain during normal development and following inactivation with Osr1-Cre

BMP signaling has been implicated in the regulation of ID PCD due to the expression pattern of BMP ligands, such as BMP 2, 4, and 7, which correlates with the initiation and progression of PCD\textsuperscript{34,42}. Thus, it is important to examine the expression of these ligands in the ID relative to Osr1-Cre activation and PCD initiation. Within this analysis, Bmp7 expression is the most robust in the ID during normal development (Figure 5G, 6H and I, 8C). It appears that by the time Osr1-Cre activity is observed throughout the ID region (Figure 2F and G) Bmp7 expression is restricted to the periphery between the ID and the condensing mesenchyme of the digital ray. However, approximately a day earlier, at E12.5 Bmp7 expression is observed throughout the entire ID mesenchyme\textsuperscript{34,42}. Bmp2 expression appears to have a similar pattern as that observed for Bmp7, however at lower levels as detected by \textit{in situ} hybridization (Figure 5A, 6A and G, 8A). On the other hand, Bmp4 expression is not detected in the ID around the time Osr1-Cre activity and PCD initiation can be observed (Figure 5D and 6E). At \textasciitilde E13.5, Bmp4 expression is mostly restricted to the sub-AER region above the digital rays.
Unlike Bmp2 and 7, there never appears to be robust Bmp4 expression in the ID mesenchyme throughout normal development\textsuperscript{34,42}. Taken together, these observations suggest that if the expression pattern of ID Bmp2, 4, and 7 during normal development correlates with the role each ligand has in regulating PCD, then BMP7 likely plays the greatest role while BMP4 might have a minimal role, if any.

4.2 Examining necessity and redundancy of interdigit BMP 2, 4, 7 in programmed cell death regulation

To examine the role of ID BMP2, 4, and 7 in regulating PCD thoroughly, these ligands were inactivated individually (Figure 5) and in various combinations using Osr1-Cre (Figures 6, 7, and 8). In the case of each gene inactivation, \textit{in situ} hybridization for the excised region was used to determine the extent of recombination during embryogenesis and confirm that the lack of a syndactylous phenotype is not due to inefficient Cre activity. Furthermore, Msx2 expression was examined to compare any observable phenotype with changes in BMP signaling within the ID mesenchyme.

First, we examined adult hindlimbs in mice where Bmp2 was inactivated with Osr1-Cre and observed complete digit separation (i.e. no syndactly/webbing) (Figure 5C). \textit{In situ} hybridization analysis for the excised exon of Bmp2 mRNA at E13.5 revealed that expression was absent from the ID mesenchyme of mutants (Osrl-Cre; Bmp2\textsuperscript{\textit{fl}}x/f\textsuperscript{\textit{lo}}x) (Figure 5A). Although most of the ID of control embryos (Bmp2\textsuperscript{\textit{fl}}x/f\textsuperscript{\textit{lo}}x) lacks Bmp2 mRNA, expression is observed at the periphery between the ID and the digital ray (Figure 5A). Furthermore, a decrease in ID Bmp2 expression did not result in a change in ID Msx2 expression (Figure 5B). However, previous work in the Tabin laboratory
demonstrates that Prx1-Cre mediated Bmp2 inactivation within the limb bud mesenchyme at E10.5 results in ID3 and ID4 soft-tissue syndactyly with variable penetrance\(^7\). Together, these results indicate that Bmp2 expression at the time of Osr1-Cre activity is not necessary during normal development for proper ID PCD.

Next, the role of ID BMP4 in the process of PCD was examined. Previous studies on limb development comparing a hypomorphic and null Bmp4 allele indicated that limb patterning is particularly sensitive to BMP4 dosage\(^1\)\(^3\),\(^1\)\(^4\). Therefore, to test whether BMP4 is necessary for ID PCD, two types of experimental crosses were used. Bmp4\(^{flox/flox}\) females were mated to either Osr1-Cre; Bmp4\(^{flox/flox}\) or Osr1-Cre; Bmp4\(^{flox/null}\) males. This assured that a lack of a phenotype is not due to a decrease in Cre efficiency in the presence of the additional flox allele necessary to be recombined for complete Bmp4 inactivation. Normal digit separation was observed in adult hindlimbs of both Osr1-Cre; Bmp4\(^{flox/flox}\) (data not shown) and Osr1-Cre; Bmp4\(^{flox/null}\) (Figure 5F) mutants. In addition, there was no detectable ID Bmp4 expression or change in Msx2 expression at E13.5 in Osr1-Cre; Bmp4\(^{flox/null}\) embryos (Figure 5D and E). Earlier inactivation of Bmp4 at ~E10.5 within the limb bud mesenchyme with Prx1-Cre also did not result in soft-tissue syndactyly\(^7\). Therefore, Bmp4 expression at the time of Osr1-Cre activity is not necessary during normal development for proper ID PCD.

To determine the role of ID BMP7 in the process of PCD, Osr1-Cre; Bmp7\(^{flox/flox}\) mice were examined. Cre positive mutant adults exhibited soft tissue syndactyly between digits two and three (ID2) and between digits three and four (ID3) (Figure 5I, red arrows). This phenotype is 100% penetrant and was observed in the forelimbs (data not shown) as well as the hindlimbs. Examining Bmp7 expression at E13.5 indicated that it
was absent in ID2 and ID3 (Figure 5G). However, some Bmp7 expression remains in ID1 along the periphery of digit two, as well as in ID 4 along the periphery of digit four (Figure 5G, dotted yellow ovals). The more extensive reduction of Bmp7 only in ID 2 and ID3 correlates with a decrease in Msx2 (Figure 5H, dotted black ovals) and the presence of soft tissue syndactyly observed only in these IDs of the adult mice (Figure 5I, red arrows). This analysis indicates that ID BMP7 is necessary for ID BMP signaling and PCD during normal development.
Figure 5. Individual inactivation of Bmp2, 4, and 7 with Osr1-Cre reveals that ID BMP7 is necessary for PCD during normal development.
**Figure 5. Individual inactivation of Bmp2, 4, and 7 with Osr1-Cre reveals that ID BMP7 is necessary for PCD during normal development.** Each of the three genes encoding the ligands BMP2, 4, and 7 were inactivated individually with Osr1-Cre to determine if they are necessary for normal ID PCD. Whole mount *in situ* hybridization using a probe specific to the floxed region of each gene demonstrates a decrease in mRNA expression of the corresponding ligand within the Osr1-Cre domain (**A**, **D** and **G**). Inactivation of the gene encoding *Bmp7* with Osr1-Cre results in a decrease in the expression of *Msx2*, a downstream target of Bmp signaling, in ID2 and ID3 (**H**, black dotted ovals). ID inactivation of the genes encoding *Bmp2* or *Bmp4*, however, did not result in a decrease of *Msx2* expression (**B** and **E**). Furthermore, the decrease in BMP signaling observed in the Bmp7 ID reduction is associated with soft tissue syndactyly within ID2 and ID3 (**I**, red arrows). Note that the lack of decrease in *Msx2* expression and syndactyly within ID1 and ID4 of Osr1-Cre; *Bmp7*\(^{\text{flox/flox}}\) mutants correlates with incomplete inactivation of *Bmp7* (**G**, yellow dotted ovals). In addition to the relevant genotypes that are shown, all experimental mice also contain a copy of *Rosa\(^{mTmG}\)* that was inherited from the mother.
To test whether BMPs play redundant roles in ID PCD, Bmp2, 4, and 7 were inactivated in combinations of two. First, Bmp2 and 4 were inactivated with Osr1-Cre. Even though, when these genes were inactivated individually the limbs developed normally, upon the compound inactivation of Bmp2 and 4 syndactyly was observed in ID2 and ID3 with variable penetrance and severity (Figure 6C, black oval). Five out of nine Osr1-Cre; Bmp2^{flox/flox}; Bmp4^{flox/flox} adults had syndactyly in ID2 and ID3 less severe than that observed in Osr1-Cre; Bmp7^{flox/flox} mutants (compare 6C to 5I). The most severe syndactyly of the five mutants is shown in Figure 6C.

Compound inactivation of Bmp4 and 7 resulted in a syndactylosous phenotype with 100% penetrance (Figure 6F). The severity of this syndactyly is greater than the compound inactivation of Bmp2 and 4, however the severity appeared similar to that of Bmp7 inactivation alone. By contrast, simultaneous inactivation of Bmp2 and 7 resulted in a syndactylosous phenotype that was consistently more severe than that of Bmp7 inactivation alone. In all of the syndactylosous mutants, webbing is observed only in ID2 and ID3 and in situ hybridization for the excised part of the corresponding transcript showed no detectable mRNA levels within ID2 and ID3 (Figure 6). This analysis indicates that although BMP7 is necessary for PCD during normal development, BMP2 and 4 do play redundant roles in the regulation of ID PCD.
Figure 6. Combinatorial inactivation of ID Bmp ligands reveals redundancies in ID PCD.
Figure 6. Combinatorial inactivation of ID Bmp ligands reveals redundancies in ID PCD. To explore redundancy of BMP ligands in ID PCD, pairs of ligands were inactivated using Osr1-Cre. Osr1-Cre inactivation of Bmp2 and Bmp4 resulted in a mild syndactylous phenotype with variable penetrance in ID2 and ID3 of adult hindlimbs (C, red arrows; 5/9 had mild syndactyly). Osr1-Cre; Bmp4\textsuperscript{flox/flox}, Bmp7\textsuperscript{flox/flox} animals displayed a moderate syndactylous phenotype in ID2 and ID3 (F, red arrows). Compound inactivation of Bmp2 and Bmp7 with Osr1-Cre resulted in severe syndactyly (I, red arrows). Whole mount in situ hybridization was performed utilizing probes containing the recombined portions of the conditionally deleted genes, to confirm loss of mRNA expression of the corresponding ligands (compare A and B, D and E, G and H). As expected, mRNA corresponding to the genetic manipulation was efficiently decreased in the ID region of Osr1-Cre expressing embryos. In addition to the relevant genotypes that are shown, all experimental mice also contain a copy of Rosa\textsuperscript{mTmG} that was inherited from the mother.
The redundancy of Bmp2 and 4 was further examined by inactivating only one copy of Bmp7 in either an Osr1-Cre; Bmp2^{flox/flox} or Osr1-Cre; Bmp4^{flox/flox} background. IDs lacking one copy of Bmp7 have normal digit separation, however, Osr1-Cre; Bmp2^{flox/flox}; Bmp7^{flox/wt} mice have ID2 and ID3 syndactyly similar to that of Osr1-Cre; Bmp7^{flox/flox} mice and less severe syndactyly than Osr1-Cre; Bmp2^{flox/flox}; Bmp7^{flox/flox} mice (Figures 7 and 6I). This suggests that the ID is sensitive to total BMP levels and corroborates that BMP2 and 7 play redundant roles in the regulation of ID PCD. However, inactivating one copy of Bmp7 in a Osr1-Cre; Bmp4^{flox/flox} background did not result in a syndactylous phenotype (data not shown). Although it is evident that both BMP2 and 4 play redundant roles in ID PCD since Osr1-Cre; Bmp2^{flox/flox}; Bmp4^{flox/flox} mutants have minor syndactyly with ~50% penetrance, this analysis suggests that BMP2 has a greater role than BMP4 in ID PCD.
Figure 7. Bmp2 inactivation sensitizes the ID mesenchyme to Bmp7 perturbation.

Inactivation of one allele of Bmp7 (A) or one allele of Bmp2 and Bmp7 (B) has no affect on ID regression, however homozygous inactivation of Bmp2 in a Bmp7 heterozygous animal causes moderate syndactyly (red arrows, C).
Since previous analyses established that all three BMP ligands 2, 4, and 7 have an effect on ID PCD, syndactyly was examined in the triple compound inactivation of $Bmp2^{\text{flox/flox}} \; Bmp4^{\text{flox/flox}} \; Bmp7^{\text{flox/flox}}$ with Osr1-Cre. Similarly, webbing was observed in both ID2 and ID3 (Figure 8). Furthermore, in situ hybridization analysis of the excised exons of $Bmp2$, $4$, and $7$ in E13.5 hindlimbs of these mutants confirmed that transcripts for these three Bmp genes were absent in ID2 and ID3 of these triple mutants (Figure 8).
Figure 8. Simultaneous inactivation of \textit{Bmp2}, 4, and 7 with Osr1-Cre.
Figure 8. Simultaneous inactivation of \textit{Bmp2}, 4, and 7 with Osr1-Cre. The triple ID ligand inactivation, Osr1-Cre; $Bmp2^{\text{flo}x/\text{flo}x}$; $Bmp4^{\text{flo}x/\text{flo}x}$; $Bmp7^{\text{flo}x/\text{flo}x}$, results in ID2 and ID3 soft-tissue syndactyly (D, red arrows). This phenotype correlates with a lack of ID2 and ID3 $Bmp2$ and $Bmp7$ mRNA expression at E13.5 (A and C). $Bmp4$ mRNA is already downregulated in the ID at this developmental point (B). In addition to the relevant genotypes that are shown all experimental mice also contain a copy of $Rosa^{mTmG}$ that was inherited from the mother.
4.3 Analysis of Bmp2, 4, 7 mutants

To further investigate how inactivating the three genes encoding the ligands BMP2, 4, and 7 within the ID mesenchyme affected BMP signaling and PCD regulation during embryonic limb development hindlimbs of ~E13.5 Osrl-Cre; Bmp2\textsubscript{flox/flox}; Bmp4\textsubscript{flox/flox}; Bmp7\textsubscript{flox/flox} and their Cre negative littermate controls were examined. LysoTracker Red was used to fluorescently mark all dying cells and assay changes in total cell death during limb development. A decrease in LysoTracker Red is observed in ID2 and ID3 of Osrl-Cre; Bmp2\textsubscript{flox/flox}; Bmp4\textsubscript{flox/flox}; Bmp7\textsubscript{flox/flox} mutants compared to their Cre negative littermate controls (Figure 9A, dotted white oval). This decrease in total cell death correlates with the syndactylous phenotype observed in adult hindlimbs (Figure 8D). The current model for PCD regulation involves an antagonistic relationship between AER-FGF as a survival factor and RA as a cell death inducer\textsuperscript{43}. Therefore, it is necessary to examine whether AER-Fgf expression and RA signaling are affected upon Osrl-Cre inactivation of Bmp2, 4, and 7. AER-FGF signaling was examined through \textit{in situ} hybridization for the Fgf8 transcript. At E13.5, AER-Fgf8 expression is appropriately downregulated above the ID mesenchyme in the mutants similarly to their littermate controls (Figure 9B white arrows). Rarβ expression was examined to determine changes in RA signaling since Rarβ is a downstream target of the RA pathway\textsuperscript{115,116}. At E13.5 there were no observable differences in ID Rarβ expression of Osrl-Cre; Bmp2\textsubscript{flox/flox}; Bmp4\textsubscript{flox/flox}; Bmp7\textsubscript{flox/flox} compared to littermate controls (Figure 9C, black dotted oval). Taken together, since no observable changes in AER-Fgf and Rarβ expression were detected, these data suggest that ID2 and ID3 syndactyly is due to a decrease in total cell death primarily resulting from Bmp2, 4, and 7 ligand inactivation.
Figure 9. ID Bmp2, 4, 7 inactivation results in a decrease of ID PCD without affecting AER-Fgf8 expression or retinoic acid signaling.
Figure 9. ID Bmp2, 4, 7 inactivation results in a decrease of ID PCD without affecting AER-Fgf8 expression or retinoic acid signaling. A decrease in cell death is observed in ID2 and ID3 upon Bmp2, 4, and 7 inactivation with Osr1-Cre (A, white dotted ovals). In these mutants AER-Fgf8 expression is appropriately downregulated above the ID mesenchyme (B, white arrows). Furthermore, ID Rarβ expression is also at the same levels as the littermate controls (C, black dotted ovals). In addition to the relevant genotypes that are shown all experimental mice also contain a copy of Rosa<sup>mTmG</sup> that was inherited from the mother.
To further examine the role of ID Bmp2, 4, and 7 in PCD during normal development, intracellular BMP signaling and cell death was further analyzed. To determine effects of BMP signaling to the ID mesenchyme, Msx2 expression and phosphorylation of the receptor SMADs 1 and 5 was examined. Msx2 has been established as a direct target of BMP signaling and its expression is often used as a readout of BMP pathway activation\textsuperscript{54,113,116}. In Osr1-Cre; Bmp2\textsuperscript{flox/flox}; Bmp4\textsuperscript{flox/flox}; Bmp7\textsuperscript{flox/flox} mutants Msx2 expression is downregulated in ID2 and ID3 confirming that BMP signaling to the ID mesenchyme is perturbed (Figure 10A). Whole mount immunohistochemistry using an antibody for the phosphorylated and activated receptor SMADs 1 and 5 further confirmed that canonical BMP signaling is affected upon Bmp2, 4, and 7 inactivation (Figure 10B). The genetic regulation of PCD can occur through several cell death mechanisms such as apoptosis, necroptosis, and autophagy\textsuperscript{117,118}. Caspase 3 is an effector caspase within the apoptotic cell death pathway and has no activity until cleaved by an initiator caspase\textsuperscript{119,120}. This active form, commonly referred to as cleaved Caspase 3, is one of the major effectors of physiological cell death in the developing limb\textsuperscript{40,119-121}. Whole mount immunohistochemistry at E13.5 using an antibody for cleaved Caspase 3 showed a dramatic decrease of activated Caspase 3 in mutants, indicating that ID Bmp2, 4, and 7 regulate apoptotic cell death (Figure 10C). Taken together, these data suggest that ID BMP2, 4, and 7 secretion from the ID signals to the ID mesenchyme through the canonical SMAD dependent cascade to activate apoptotic cell death.
Figure 10. A decrease in cleaved Caspase 3, Msx2, and phosphorylated SMAD 1 and 5 is observed in Osr1-Cre Bmp2, 4, and 7 mutants.
Figure 10. A decrease in cleaved Caspase 3, Msx2, and phosphorylated SMAD 1 and 5 is observed in Osr1-Cre Bmp2, 4, and 7 mutants. Upon ID Bmp2, 4, and 7 inactivation a decrease in Msx2 expression (A) and receptor SMAD 1, 5 phosphorylation (B) is associated with a decrease in apoptotic cell death, as detected through cleaved Caspase 3(C). In addition to the relevant genotypes that are shown all experimental mice also contain a copy of Rosa<sup>mTmG</sup> that was inherited from the mother.
4.4 Summary

To determine whether ID BMP ligand secretion regulates PCD, ID tissue-specific mesenchymal Osr1-Cre was used to inactivate Bmp2, 4, and 7 in various combinations. It was determined that ID Bmp7 expression is necessary for PCD during normal development. Furthermore, Bmp2 and 4 have redundant roles, of which Bmp2 inactivation appears to have a greater effect on ID syndactyly than Bmp4. Analysis of Osr1-Cre:Bmp2\textsuperscript{flox/flox},Bmp4\textsuperscript{flox/flox},Bmp7\textsuperscript{flox/flox} E13.5 hindlimbs revealed that a decrease in PCD results in a syndactylous phenotype while the normal antagonistic relationship between AER-FGF and retinoic acid signaling remains unperturbed. Further analysis revealed a decrease in BMP signaling to the ID mesenchyme as observed through a decrease in Msx2 expression and in SMAD 1 and 5 phosphorylation. Finally, loss of ID BMP signaling is associated with a decrease in ID cleaved Caspase 3 and thus, apoptotic PCD.

4.5 Discussion

Previously published work showed that inactivating Bmp2 and Bmp4 by Prx1-Cre in the mesenchyme results in a decrease of cell death and soft tissue syndactyly, thus suggesting that ID BMP ligands redundantly regulate ID PCD\textsuperscript{70}. However, an abnormal retention of Fgf8 above the ID mesenchyme is also observed in the Prx1-Cre; Bmp2; Bmp4 compound mutants\textsuperscript{70}. Thus, in the context of Prx1-Cre inactivation of Bmp2 and Bmp4, the resulting syndactyly could be due to the increase in AER-Fgf8 expression\textsuperscript{42}. Therefore, the tissue-specific inactivation of Bmp2, 4, and 7 in the ID using Osr1-Cre is critical in revealing how PCD is regulated during normal development. Since the AER-
FGF and retinoic acid signaling pathways are unperturbed, this indicates that pSMAD dependent BMP signaling from the ID mesenchyme is necessary for PCD during normal development. Furthermore, the appropriate downregulation of AER-\textit{Fgf8} expression above the ID mesenchyme suggests, that at this developmental time point, BMP2, 4, and 7 secreted from the ID mesenchyme do not signal to the AER and regulate \textit{Fgf8} expression. Although it is known that BMP signaling within the AER through the BMP ligands 2, 4, and 7 and BMPR1A is necessary for appropriate AER-\textit{Fgf} regulation, it is still unclear whether ID BMP ligand expression could also play a role in AER-FGF regulation\textsuperscript{42,71,107}. Since appropriate downregulation of AER-\textit{Fgf8} expression is observed upon inactivation of the genes encoding \textit{Bmp2}, 4, and 7 with Osr1-Cre, this suggests that at the time of Osr1-Cre activity initiation, \textit{Bmp2}, 4, and 7 expression from the ID mesenchyme does not regulate AER-\textit{Fgf8} expression. In addition, of the three BMP ligands, only BMP 7 is necessary for PCD to proceed normally during development. Although previously published BMP7 deletions have affected limb development, the Osr1-Cre tissue specific inactivation has allowed for BMP7’s role in ID PCD to be revealed since this manipulation is not lethal and does not result in skeletal phenotypes\textsuperscript{70}. Finally, these analyses and previously published work indicate that ID PCD regulation through AER-FGFs, ID retinoic acid signaling, and ID BMP signaling all need to be appropriately regulated for limb development and morphogenesis to proceed normally during embryogenesis.
CHAPTER 5. BMP SIGNALING DIRECTLY REGULATES INTERDIGIT PROGRAMMED CELL DEATH
Studies on early limb development implicated BMPs as the effectors and initiators of ID PCD\(^48,57,70,73\). However, because it was elucidated that BMP signaling in the AER indirectly regulates ID PCD through modulating \(Fgf\) expression, BMPs have been generally dismissed as direct regulators of ID PCD\(^42,43\). In fact, the current and predominant model for ID PCD is based on an antagonistic relationship between AER-FGF and ID RA signaling that dictates the precise timing of PCD initiation\(^43,122\). Furthermore, genetic analysis using Cre tissue-specific inactivation in the AER provided evidence that SMAD1/5 dependent AER-BMP signaling initiated by BMP2, 4, and 7 ligand binding indirectly regulates ID PCD through modulating AER-FGF secretion to the underlying mesenchyme\(^42,71,107,121\). Currently there is no genetic evidence available that addresses whether BMP signaling to the ID mesenchyme directly regulates PCD without affecting AER-FGF secretion or RA metabolism. In the previous chapter, the ID specific inactivation of \(Bmp2\), 4, and 7 revealed that ID BMP ligand gene function is involved in the regulation of PCD. However, this regulation could still comprise an indirect signaling cascade that has not been examined. To determine whether BMPs are direct effectors of PCD, BMP signaling selectively to the ID mesenchyme needs to be abolished.

5.1 Interdigit BMP signaling through BMPR1A is necessary for programmed cell death

To test whether BMP signaling to the ID mesenchyme directly regulates PCD Osr1-Cre was used to inactivate \(Bmpr1a\). The hindlimbs and forelimbs of the resulting mutants have soft tissue syndactyly that is most severe in the anterior of the autopod.
(Figure 11B, red arrows). This phenotype is 100% penetrant and, in the hindlimb, webbing is most severe within ID1, extending to the distal tip of digit one. Although webbing is observed in the forelimb, due to the lack of digit separation during normal development between digits one and two, the severity of ID1 syndactyly cannot be assessed in adults. In both forelimbs and hindlimbs, syndactyly is also observed within ID2 and ID3, and sometimes in ID4. An examination during embryonic development at ~ E13.5 revealed a decrease in total cell death, most apparent within ID1, as observed via LysoTracker Red fluorescence (Figure 11F). Since BMPs have been shown to affect PCD indirectly through AER-FGFs, it has to be determined whether the antagonistic balance between AER-Fgf expression and RA signaling is affected. In Osr1-Cre; Bmpr1a^floxt/null mutants AER-Fgf8 expression is appropriately down regulated above the ID mesenchyme comparable to that of littermate controls (Figure 12B, black arrow). To assess retinoic acid signaling, mRNA expression of the downstream target, Rarβ, was examined. Normal Rarβ expression was observed throughout all four IDs of Osr1-Cre; Bmpr1a^floxt/null mutant mice (Figure 12F). Taken together, these data indicate that BMP signaling to the ID mesenchyme directly regulates PCD in a cell autonomous manner.

5.2 BMPR1B plays a redundant role in apoptotic interdigit programmed cell death

Bmpr1b is an additional type 1 BMP receptor expressed in the limb mesenchyme. Furthermore, BMPR1A and BMPR1B have been shown to play redundant roles in development, in addition to limb morphogenesis\textsuperscript{123,124}. For example, BMPR1A and BMPR1B act redundantly during tooth and plate development and chondrogenesis of the limb skeletal elements\textsuperscript{123,124}. To test whether these two type 1 receptors also have a
redundant role in ID PCD, Bmpr1a was inactivated with Osr1-Cre in a Bmpr1b null background. The BMPR1B mutants have been previously characterized and have skeletal malformation of the autopod\textsuperscript{89}. Bmpr1b null mice have brachydactyly (short digits) due to a defect in chondrogenesis of the proximal phalanges (Figure 11C). Nonetheless, the short digits of the BMPR1B mutants are completely separated and there is no soft tissue syndactyly, indicating that BMPR1B is not necessary for PCD during normal development. Syndactyly can be observed in mutants with both Bmpr1a and Bmpr1b inactivated in the ID, but the skeletal malformations of the autopod make it confounding to determine if these mutants have a more severe syndactylous phenotype than the sole inactivation of Bmpr1a. Therefore, LysoTraker red was used to assess PCD at E13.5 when the brachydactyly phenotype does not limit further analysis. At E13.5, total cell death is even further decreased in mutants with compound Bmpr1a and Bmpr1b ID inactivation (Figure 11H) than the inactivation of Bmpr1a alone (Figure 11F). In addition, AER-Fgf8 expression is appropriately downregulated above the ID mesenchyme in Bmpr1b null embryos and in mutant embryos with both ID Bmpr1a and Bmpr1b inactivated (Figure 12 C and D) suggesting that AER-FGF regulation of PCD is not indirectly affected. Similarly, Rarβ expression is at wildtype levels in Bmpr1b null embryos and Bmpr1a and Bmpr1b compound mutants (Figure 12 G and H). Therefore, the even greater decrease of PCD in the compound ID inactivation of Bmpr1a and Bmpr1b indicates that they have redundant roles in the regulation of ID PCD. Whole mount immunohistochemistry for the activated cleaved Caspase 3 revealed that inactivating Bmpr1a results in a decrease of apoptotic cell death (Figure 11J), which is most apparent in the anterior ID. This decrease in apoptotic PCD is also observed when
Bmpr1a is inactivated with Osr1-Cre in the Bmpr1b null background (Figure 11L). Overall, this analysis reveals that Bmpr1a is necessary for and directly regulates ID apoptosis without affecting the AER-Fgf and RA antagonistic relationship, while Bmpr1b can act redundantly in ID PCD.
Figure 11. ID Bmpr1a is necessary for apoptotic PCD during limb development while Bmpr1b plays a redundant role
Figure 11. ID Bmpr1a is necessary for apoptotic PCD during limb development while Bmpr1b plays a redundant role. To test the cell autonomous PCD regulation of ID BMP signaling, Bmpr1a was inactivated with Osr1-Cre. At P22 the hindlimbs of Osr1-Cre; Bmpr1a\textsuperscript{flox/null} mutants have soft tissue syndactyly (B, red arrows) in ID1 through ID3 with 100% penetrance. Syndactyly extends to the distal tip of digit one. E13.5 hindlimbs of Osr1-Cre; Bmpr1a\textsuperscript{flox/null} mutants have a decrease in total cell death, as detected by LysoTracker Red, which is most apparent in ID1 (F). To test redundancy in the regulation of PCD, ID Bmpr1a was inactivated in a Bmpr1b null background. Bmpr1b controls (C) have brachydactyly but no soft tissue syndactyly. The Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} compound mutants have syndactyly most severe in ID1 (D). At E13.5, loss of total cell death is greater in the compound Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} mutant (H) when compared to the ID inactivation of just Bmpr1a (F). Furthermore, an apparent decrease of activated cleaved Caspase 3 is observed in a similar pattern to changes in LysoTracker of the syndactylyous mutants (compare J and L to I and K, black ovals).
Figure 12. Osr1-Cre Bmpr1 inactivation results in a decrease of Msx2 and pSMAD 1, 5 without affecting AER-Fgf8 and retinoic acid signaling.
Figure 12. Osr1-Cre Bmpr1 inactivation results in a decrease of Msx2 and pSMAD 1, 5 without affecting AER-Fgf8 and retinoic acid signaling. To confirm that the antagonistic relationship between AER-FGF and retinoic acid signaling is not perturbed

Fgf8 (A-D) and Rarβ (E-F) were examined. AER-Fgf8 expression is appropriately downregulated above the ID mesenchyme in both syndactylyous mutants (B and D, black arrows) and controls (A and C). Similarly, there is no apparent difference in Rarβ expression between the Osr1-Cre; Bmpr1a<sup>flox/null</sup> and Osr1-Cre;Bmpr1a<sup>flox/null</sup>; Bmpr1b<sup>null/null</sup> mutants (F and H) and the corresponding controls (E and G). Next SMAD dependent BMP signaling was examined. A decrease in the transcript of the BMP downstream target, Msx2, indicates that ID BMP signaling has been perturbed in both syndactylyous mutants (J and L, black ovals) compared to littermate controls (I and K).

Whole mount immunofluorescence revealed a decrease in phosphorylated SMAD 1 and 5 just in the distal ID domain (N and P), most apparent in ID1 (yellow ovals). Phosphorylated SMAD 1 and 5 is still detectable in the proximal ID regions (N and P).
5.3 Analysis of the cell autonomous ID Bmpr1 inactivation

To examine how abolishing BMP signaling to the ID mesenchyme regulates PCD whole mount *in situ* hybridization and immunofluorescence was performed at E13.5, when the earliest effects on total cell death are apparent. To examine the effects on BMP signaling after *Bmp* receptor inactivation in the ID mesenchyme, *Msx2* transcription, a target and readout of BMP signaling, and receptor SMAD 1 and 5 phosphorylation were examined. In *Bmpr1a* mutants *Msx2* transcription is decreased in ID1 through ID3 compared to littermate controls (Figure 12J, black oval). Nonetheless, some *Msx2* expression still remains in ID1 through ID4, which could be from RNA perdurance after Cre activation, heterogeneous *Bmpr1a* inactivation throughout the ID mesenchyme, or BMP signaling through other ID BMP receptors. Surprisingly, in *Bmpr1b* null mutants, an overall elevation in *Msx2* expression is observed in the ID region (Figure 12K). However, upon compound inactivation of *Bmpr1a* with Osr1-Cre in a *Bmpr1b* null background, a decrease in *Msx2* expression is observed from ID1 through ID3 (Figure 12L, black oval). Note that the pattern of *Msx2* expression with Osr1-Cre is similar to that of Osr1-Cre activity – the greatest decrease in *Msx2* expression is observed in the anterior of the limb while ID4 has wildtype levels or slightly decreased expression levels (Figure 12 I-L). The decrease in PCD of Osr1-Cre; *Bmpr1a; Bmpr1b* compound mutants is also associated with a decrease in ID *Msx2* expression (Figure 12L).

Upon initiation of the BMP signaling cascade through BMPR1A activation, receptor SMADs 1, 5, and 8 are recruited and activated through a phosphorylation event. Thus, to examine the effect of ID *Bmp* receptor inactivation on canonical BMP signaling, phosphorylated SMAD 1 and 5 were examined via whole mount immunofluorescence at
E13.5 (Figure 12, M-P). Although a decrease in phosphorylated SMAD 1 and 5 is observed in the distal domains of ID1 through ID3 upon Osr1-Cre Bmpr1a inactivation, there does not appear to be a change in SMAD 1 and 5 phosphorylation in the proximal ID domains, where the most significant decrease in PCD is observed. This suggests that BMP signaling to the ID mesenchyme could regulate apoptosis through both a SMAD independent and a SMAD dependent mechanism.

5.4 Role of interdigit BMP receptors in senescence, vascular patterning, and levels of reactive oxygen species

Recently, the potential roles of senescence and vascular patterning in limb morphogenesis during normal development have been elucidated. Thus, we examined the effects of Bmp receptor inactivation on ID senescence and vascular patterning at ~E13.5.

It has been suggested that senescence is a part of normal limb development and could be a necessary step to recruit macrophages to sites of cell death prior to initiation of apoptosis. These non-proliferative cells are detected through their senescence – associated β-galactosidase (SA-βgal) activity. Similar to apoptotic ID PCD, a decrease in SA-βgal activity is observed in Osr1-Cre; Bmpr1a\(^{\text{flox/null}}\) and Osr1-Cre; Bmpr1a\(^{\text{flox/null}}\); Bmpr1b\(^{\text{null/null}}\) mutants (Figure 13 B and D) as compared to littermate controls (Figure 13 A and C, respectively). This suggests that ID Bmp receptor inactivation results in a decrease of ID senescence that correlates with a decrease in ID apoptosis.

Previous research has demonstrated that, prior to PCD initiation an increase in ID vasculature is observed. Genetic manipulations either increasing or decreasing ID
vasculature demonstrated that the limb vasculature positively regulates ID PCD, likely through levels of reactive oxygen species (ROS)\textsuperscript{77}. This suggests that tissue oxygenation is necessary for ROS production and proper PCD initiation\textsuperscript{77}. However, it is still unclear whether the known genetic regulation of PCD is also involved in vascular patterning and generation of ROS. To determine levels of ROS, Dihydroethidium (DHE) was utilized as previously described\textsuperscript{77}. Similar to the pattern of apoptotic PCD, a decrease in ROS was observed in both Osr1-Cre; \textit{Bmpr1a}\textsuperscript{flx/null} and Osr1-Cre;\textit{Bmpr1a}\textsuperscript{flx/null}; \textit{Bmpr1b}\textsuperscript{null/null} mutants (Figure 13 F and H) as compared to littermate controls (Figure 13 E and G, respectively). Furthermore, a decrease in ROS was also observed in Msx2-Cre; \textit{Bmpr1a}\textsuperscript{flx/null} mutants (data not shown). Therefore, both ID-BMP and AER-FGF signaling regulate ROS levels. However, PECAM immunofluorescence revealed no change in ID vascular patterning in neither \textit{Bmpr1a} inactivation in the ID with Osr1-Cre (Figure 14) or AER with Msx2-Cre (data not shown). Therefore, regulation of ID vascular patterning is upstream of ID-BMP and AER-FGF signaling and ROS levels are not directly dependent on the extent of the vascular network.
Figure 13. A decrease in senescence and reactive oxygen species is observed upon ID Bmpr1 inactivation. Senescence associated β-galactosidase (SA-βgal) staining reveals a decrease in senescing cells upon Bmpr1a inactivation with Osr1-Cre in a wildtype (B) and Bmpr1b null background (D) compared to littermate controls (A and C). This difference in SA-βgal has a similar pattern to the observed changes in LysoTracker Red, with the greatest decrease within ID1 (black ovals) and most apparent in Osr1-Cre; Bmpr1a<sup>flox/null</sup>; Bmpr1b<sup>null/null</sup> compound mutants (D). Levels of reactive oxygen species (ROS) were examined utilizing Dihydroethidium (DHE) fluorescence (E-H). A similar decrease in ROS levels upon Bmpr1a inactivation with Osr1-Cre in a wildtype (F) and Bmpr1b null background (H) compared to littermate controls (A and C) was observed.
Figure 14. Normal vascular patterning is observed in Osr1-Cre; Bmpr1α<sup>flox/null</sup> syndactylous mutants. PECAM whole mount immunofluorescence was utilized to examine ID vascular patterning in Osr1-Cre; Bmpr1α<sup>flox/null</sup> syndactylous mutants at E13.5. No apparent difference in the ID vascular network was observed between mutants and controls.
5.5 Potential role of SMAD independent signaling in the regulation of interdigital programmed cell death

There are several alternate ways that BMP signaling could be propagated using SMAD independent mechanisms\textsuperscript{50,125-127}. These include the phosphorylation of ERK, p38, JNK, or AKT, as well as the transcriptional activation of Dkk1\textsuperscript{50,125-128}. In particular, Dkk1 transcription has been shown to be regulated by SMAD independent BMP signaling through p38 phosphorylation during skeletogenesis in mice, and through BMP5 induced ID PCD in chick\textsuperscript{50,128}. \textit{In situ} hybridization revealed a decrease in ID Dkk1 transcripts in Osr1-Cre; Bmpr1a\textsuperscript{flox/null} mutants (Figure 15). This further supports that the regulation of ID PCD through BMPR1A also involves a SMAD independent mechanism.

Co-receptor SMAD4 is a common factor between BMP signaling and the TGF-\beta superfamily and is necessary for translocation of receptor SMADs to the nucleus so that target gene activation can occur\textsuperscript{38,44,54,129}. Global SMAD4 inactivation is lethal during early embryogenesis (E6-E7) due to malformations of the extra-embryonic membrane and a decrease in epiblast proliferation\textsuperscript{130,131}. The generation of homozygous mice with loxP sites flanking exon 8 of Smad4 allows for the tissue-specific inactivation of Smad4 at later embryonic stages\textsuperscript{97}. Furthermore, the ID tissue-specific inactivation of Smad4 with Osr1-Cre allows for an additional way to test whether ID PCD regulation could involve a SMAD independent mechanism. To accomplish this, Smad4\textsuperscript{flox/flox} females were crossed to Smad4\textsuperscript{flox/null} males that also carry one copy of the Osr1-Cre transgene. Then, the hindlimbs of adult mice were examined for the presence of ID tissue (webbing). The hindlimbs of Osr1-Cre; Smad4\textsuperscript{flox/null} adult mice had normal digit separation and no
observable ID tissue when compared to littermate controls. This is consistent with the initiation of ID PCD through a SMAD independent mechanism.
Figure 15. A decrease in ID $Dkk1$ expression in Osr1-Cre; $Bmpr1a^{flox/null}$ mutants suggests involvement of SMAD independent BMP signaling. $Dkk1$ expression was examined as a marker of SMAD independent ID BMP signaling. I decrease in proximal $Dkk1$ expression in the IDs of Osr1-Cre; $Bmpr1a^{flox/null}$ mutants is observed.
Figure 16. Lack of syndactyly in Osr1-Cre; Smad4\textsuperscript{flox/null} mice suggests involvement of SMAD independent regulation of ID BMP signaling. The adult limbs of Osr1-Cre; Smad4\textsuperscript{flox/null} mice are indistinguishable from those of littermate controls, have complete digit separation, and lack any apparent ID webbing.
5.6 Inactivation of Bmpr1a in the AER and interdigit further decrease interdigit programmed cell death

Through previous research and the presented results, it is evident that BMP signaling through BMPR1A directly regulates PCD within the ID, while indirectly regulating PCD through modulating AER-Fgf expression\textsuperscript{42}. Furthermore, it appears that, for appropriate ID PCD to occur, both a downregulation of AER-Fgf expression and ID-BMP activation is necessary. However, it is unknown whether the individual perturbation of AER-FGF or ID-BMP signaling has a similar effect on ID PCD or whether the FGF and BMP pathways exert a combinatorial effect. To test this, Bmpr1a was inactivated with Osr1-Cre and Msx2-Cre (Figure 17). As expected and previously described, a decrease in cell death is observed in Msx2-Cre; Bmpr1a\textsuperscript{flx/null} mutants (Figure 17C)\textsuperscript{42}. Interestingly, an even greater decrease in ID PCD is observed upon Bmpr1a inactivation with both Osr1-Cre and Msx2-Cre (Figure 17D). This provides evidence that the AER-FGF and the ID-BMP signaling pathways together regulate PCD.
Figure 17. Inactivation of Bmpr1a with Msx2-Cre and Osr1-Cre further decrease ID PCD. To determine whether the AER-FGF and ID-BMP signaling pathways could interact in a combinatorial manner to regulate ID PCD, Bmpr1a was inactivated with Osr1- and Msx2-Cre. In the compound Osr1-Cre; Msx2-Cre; Bmpr1a<sup>flox/null</sup> mutants an even greater decrease in LysoTracker Red is observed (D) than Msx2-Cre; Bmpr1a<sup>flox/null</sup> mutants (C). Littermate controls have robust cell death throughout all four interdigits (A and B).
5.7 Summary

To determine whether BMP signaling to the ID mesenchyme regulates PCD, Osr1-Cre was utilized to inactivate Bmpr1a alone and in a Bmpr1b null background. ID inactivation of only Bmpr1a results in a syndactylous phenotype in adults and a decrease in apoptotic cell death, senescence, and ROS levels at E13.5. Although Bmpr1b mutants do not have a syndactylous phenotype, ID inactivation of Bmpr1a in a Bmpr1b null background results in a further decrease of apoptotic ID cell death, senescence, and ROS levels. In contrast, normal levels of AER-Fgf8 and ID Rarβ expression are observed in both Osr1-Cre;Bmpr1a^{flox/null} embryos and Osr1-Cre; Bmpr1a^{flox/null}; Bmpr1b^{null/null} mutants at E13.5. While the syndactylous phenotype and decrease in cell death are associated with a downregulation of ID Msx2 expression, SMAD 1 and 5 phosphorylation is decreased only in the distal region of the ID mesenchyme. Proximal ID SMAD 1 and 5 phosphorylation appears to be unperturbed. Furthermore, Dkk1 expression is downregulated in the proximal ID of Osr1-Cre; Bmpr1a^{flox/null} mutants and inactivation of Smad4 with Osr1-Cre does not affect ID regression as evidenced by the complete digit separation and lack of syndactyly in adult Osr1-Cre; Smad4^{flox/null} mice. In addition, vascular patterning appears to be unperturbed upon Osr1-Cre inactivation of Bmpr1a. Finally, inactivation of Bmpr1a in both the AER and ID revealed that Osr1-Cre; Msx2-Cre; Bmpr1a^{flox/null} mutants have an even greater decrease in ID PCD than Msx2-Cre; Bmpr1a^{flox/null} littermates.
5.8 Discussion

The ID inactivation of *Bmpr1a* with tissue-specific Osr1-Cre provides genetic evidence that BMP signaling to the ID mesenchyme directly, and cell autonomously, regulates apoptotic PCD during normal development. The established model for ID PCD initiation involves an antagonistic relationship between inhibitory FGFs secreted from the AER and activation through ID retinoic acid metabolism\(^4^3\). BMPs are currently thought to affect ID PCD only indirectly through the regulation of *Fgf* expression in the AER\(^4^2,^4^3\). However, analysis of Osr1-Cre; *Bmpr1a*\(^{flax/null}\) and Osr1-Cre; *Bmpr1a*\(^{flax/null}\), *Bmpr1b*\(^{null/null}\) mutants indicated that BMP signaling to the ID regulates apoptosis without affecting AER-*Fgf8* expression or ID retinoic acid signaling. Therefore, during normal development, ID BMP signaling directly regulates PCD independent of AER-FGFs or retinoic acid. In addition, direct regulation of ID PCD by BMPs also correlates with a decrease in ID senescence and levels of reactive oxygen species. Moreover, an examination of the limb vasculature via PECAM whole mount immunofluorescence indicated that the genetic regulation of ID ROS levels and PCD is downstream of vasculogenesis during normal limb morphogenesis.

BMPs’ direct regulation of ID PCD could involve both a SMAD dependent and independent signaling cascade. It was previously published that E13.5 embryonic limbs cultured with Dorsomorphin, a small molecule inhibitor of SMAD dependent BMP signaling, had normal apoptotic cell death\(^4^3,^7^8\). This indicates, that in the absence of SMAD dependent signaling an alternate BMP pathway could regulate PCD. Consistent with this interpretation, no change in SMAD 1 and 5 phosphorylation was observed in the proximal ID region where the greatest decrease in ID PCD is observed in mutants with
inactive \textit{Bmpr1a}. Nonetheless, the decrease in pSMAD 1 and 5 in the distal most part of ID1 through ID3 and the significant decrease of anterior ID \textit{Msx2} expression in the syndactyious mutants, clearly indicate the direct involvement of ID BMP signaling in the regulation of PCD.

To further examine whether ID SMAD signaling is involved in PCD, the co-receptor \textit{Smad4} was inactivated with Osr1-Cre. Hindlimbs of mutant animals developed normally without any ID tissue, further corroborating SMAD independent BMP regulation of ID PCD. However, to be certain that \textit{Smad4} expression and SMAD4 activity has been abolished within the ID mesenchyme, further analysis of Osr1-Cre; \textit{Smad4}\textsuperscript{flox/null} mutants is necessary. In fact, there are several instances that \textit{Smad4} inactivation with a particular Cre results in a less severe phenotype than \textit{Bmpr1a} inactivation with the same Cre\textsuperscript{42,123,132,133}. For example, Msx2-Cre; \textit{Bmpr1a}\textsuperscript{flox/null} mice do not develop hindlimbs whereas Msx2-Cre; \textit{Smad4}\textsuperscript{flox/null} mutants do, although malformed\textsuperscript{42,132}. However, molecular analysis of E9.5 limb buds of Prx1-Cre; \textit{Smad4}\textsuperscript{flox/null} embryos suggests that the \textit{Smad4} transcript and protein product are labile and perdurance of neither is observed shortly after Prx1-Cre expression\textsuperscript{134}.

While analyses of Osr1-Cre; \textit{Bmpr1a}\textsuperscript{flox/null} mutants implies that both SMAD dependent and independent BMP pathways regulate ID PCD, the specific SMAD independent mechanism that initiates ID apoptosis is still unclear. In addition to receptor SMADs, the p38 dependent regulation of \textit{Dkk1} has been demonstrated both in skeletogenesis and BMP5 activated ID PCD\textsuperscript{50,128}. Therefore, the decrease in \textit{Dkk1} in Osr1-Cre; \textit{Bmpr1a}\textsuperscript{flox/null} E13.5 embryos suggests that ID BMPs might be regulating cell death through p38 phosphorylation. Nonetheless, further characterization of Osr1-Cre;
Bmpr1a\textsuperscript{flx/null} mutants is necessary to determine the SMAD independent signaling cascade through which BMPs could regulate ID apoptosis.
CHAPTER 6: EVALUATING THE ROLE OF THE LIMB INTERDIGIT TISSUE AS A SIGNALING CENTER NECESSARY FOR SKELETOGENESIS AND DIGIT OUTGROWTH DURING NORMAL DEVELOPMENT
During embryonic limb development the homogenous mesenchymal cells of the autopod differentiate into Sox9 positive condensations separated by Sox9 negative ID mesenchyme. As the autopod further develops, the digital rays proliferate and undergo chondrogenesis while the ID mesenchyme is removed through programmed cell death (PCD). Although the ID is only transiently present during development and ultimately removed, it could potentially act as a signaling center necessary for digit formation. In particular, manipulations of chick ID mesenchyme determined that BMP secretion from the ID tissue signaled to a region at the distal tip of the condensed mesenchyme known as the phalanx forming region. Furthermore, these studies revealed that the ID signaled to the forming digit directly anterior of it and each ID had unique BMP levels that correlated with a particular digit identity. The availability of the ID tissue-specific Osr1-Cre makes it possible to genetically test whether BMP signaling around the time of PCD initiation plays a role in digit development of the autopod.

6.1 Interdigit Bmp 2, 4, 7 inactivation does not affect digit formation and skeletogenesis

Chick limb development studies suggested BMP signaling from the ID tissue regulates digit identity. For example, the placement of Noggin beads in the ID tissue of developing chick led to a change in the number of phalanges in the digit anterior to the manipulated ID tissue. Since Noggin inhibits BMP signaling by binding and sequestering BMP ligands, a similar approach would be the tissue-specific inactivation of BMP ligands secreted from the ID mesenchyme. To accomplish this, Bmp2, 4, and 7 were inactivated with Osr1-Cre and the adult hindlimb skeleton was examined for
abnormalities. Skeletal preparations of mutant hindlimbs (Osr1-Cre; Bmp2\textsuperscript{flox/flox}; Bmp4\textsuperscript{flox/flox}; Bmp7\textsuperscript{flox/flox}) appeared normal when compared to littermate controls (Bmp2\textsuperscript{flox/flox}; Bmp4\textsuperscript{flox/flox}; Bmp7\textsuperscript{flox/flox}) (Figure 18). All digits had the appropriate number of phalanges (two for digit one and three for digits two through five) and all skeletal elements appeared to be of similar length. Therefore, BMP2, 4, and 7 ligand secretion from the ID mesenchyme at approximately the time of Osr1-Cre activity (~E13.0 – E13.75) is not necessary for digit formation and identity during normal mouse limb development.
Figure 18. *Bmp2*, *4*, and *7* inactivation with *Osrl-Cre* does not result in any apparent skeletal abnormalities. To determine whether ID BMP ligand secretion affects digit formation and identity, *Bmp2*, *4*, and *7* were inactivated with Osrl-Cre and skeletal preparations of mutant adult hindlimbs were compared to Cre negative littermate controls. All digits had the appropriate number of phalanges and the skeletal elements appeared to be of similar length as the Cre negative controls.
6.2 Interdigit \textit{Bmpr1a} inactivation in \textit{Bmpr1b} null mutants rescues digit one outgrowth

\textit{Bmpr1b} null mice have skeletal abnormalities limited to the autopod that were previously characterized\textsuperscript{91,135}. The most apparent defect is that the proximal phalanx in digit one and the proximal and middle phalanges in digits two through five fail to form. Instead a rudimentary structure is observed (e.g. compare Figure 19 E and G, red and yellow ovals). Remarkably, this failure in digit outgrowth is completely rescued in digit one when \textit{Bmpr1a} is inactivated within the ID mesenchyme in \textit{Bmpr1b} null mice (compare Figure 19 E, G, and H, red ovals). It is important to note that the appendicular skeletal elements of Osr1-Cre; \textit{Bmpr1a}\textsuperscript{floX/null} mice appear normal. Nonetheless, the hindlimbs of these animals do exhibit severe ID soft tissue syndactyly which constrains the degree to which the digits can freely extend and thus they appear bent in X-ray images (Figure 19, B and F).

To quantify the extent of the observed rescue in Osr1-Cre; \textit{Bmpr1a}\textsuperscript{floX/null}; \textit{Bmpr1b}\textsuperscript{null/null} mice, the length of skeletal elements was determined from digitized X-ray images of hindlimbs from three individual animals (units in pixels). The most distal phalanx develops normally in \textit{Bmpr1b} null mutants\textsuperscript{91} and thus was excluded from measurements of digit length; only the proximal two phalanges were measured (Figure 19I). The metatarsals were similarly measured for each genotype (Figure 19J) and the mean and standard error was calculated and compared for statistical significance using an unpaired two-tailed t-test. It is clear that digits one through five of the brachydactylous mutant, Osr1-Cre; \textit{Bmpr1b}\textsuperscript{null/null} (green), are much shorter compared to control digits (blue) (Figure 19I). This decrease in digit length is completely rescued in digit one in
the compound Osr1-Cre; \( Bmpr1a^{\text{flox/null}}; Bmpr1b^{\text{null/null}} \) (purple) and there is no statistical difference when compared to control (blue) (p-value = 0.525). Although a qualitative improvement in skeletogenesis of digits two through five is observed in the double mutants via a close examination of the bone thickness of the remaining rudimentary phalanx (compare yellow ovals Figure 19 E-H), measurements reveal that there is no significant rescue in digit outgrowth. In fact, there is no statistical difference between the brachydactylous \( Bmpr1b \) null mutants and the Osr1-Cre; \( Bmpr1a^{\text{flox/null}}; Bmpr1b^{\text{null/null}} \) mice when the lengths of digits two, three and five are compared. Upon comparing digit length of mice with only \( Bmpr1a \) inactivated in the ID to control animals there appears to be a statistical difference for digits one through three (digit one p-value = 0.014, digit two p-value = 0.036, digit three p-value = 0.045) while there is no statistical difference in length of digits four and five. This variation in length is likely due to constraints in accurate measurements caused by the bending of digits one through three due to the severe sydactyly observed in Osr1-Cre; \( Bmpr1a^{\text{flox/null}} \) mutants.

Since a decrease in metatarsal length was reported in the original characterization of \( Bmpr1b \) mutants\(^9\), metatarsal length was also examined in all four genotypes (Figure 19J). In the context of this analysis, there was no statistical difference in length between \( Bmpr1b \) mutants and controls of metatarsals one and five, while a statistical difference was observed in metatarsal two through four (metatarsal two p-value=0.043, metatarsal three p-value=0.047, metatarsal four p-value=0.015). This difference in length was not rescued in the compound Osr1-Cre; \( Bmpr1a^{\text{flox/null}}; Bmpr1b^{\text{null/null}} \) mutants. Furthermore, while a statistical difference was observed in metatarsal one length of Osr1-Cre; \( Bmpr1a^{\text{flox/null}} \) mutants compared to controls (p-value=0.036), no statistical difference was
observed in metatarsals two through four. Finally, there is no statistical difference in metatarsal one length between the compound mutant and Osr1-Cre; Bmpr1a\textsuperscript{flx/wt} controls.

Overall, this analysis determined that ID Bmpr1a inactivation in Bmpr1b null mice completely rescues the brachydactyly phenotype in digit one of Bmpr1b mice indicating that BMP signaling in the ID mesenchyme plays a role in digit formation and skeletogenesis. Furthermore, an examination of other syndactylous phenotypes in a Bmpr1b background reveals that retention of the ID tissue alone is not sufficient for a rescue in digit outgrowth. For instance, inactivating Bmpr1a in the apical ectodermal ridge (AER) with Msx2-Cre results in a syndactylous phenotype due to a spatio-temporal increase of AER-Fgfs\textsuperscript{42}. However, inactivating Bmpr1a with Msx2-Cre in a Bmpr1b background does not rescue the brachydactyly phenotype observed in Bmpr1b mutants (data not shown). Inactivating the genes for the apoptotic factors BAK and BAX leads to ID webbing and no skeletal malformations\textsuperscript{136} (Figure 22B). To test whether the retention of ID tissue due to Bak and Bax inactivation could rescue brachydactyly, skeletal preparations of Bak\textsuperscript{null/null}; Bax\textsuperscript{null/null}; Bmpr1b\textsuperscript{null/null} neonates were examined (Figure 22). The brachydactyly phenotype was not rescued in these triple mutants (Figure 22 C-E).

Therefore, this indicates that the retention of ID tissue is not enough to rescue brachydactyly in Bmpr1b\textsuperscript{null/null} mutants and, specifically, BMP signaling from the ID mesenchyme likely plays a role in skeletogenesis during normal development.
Figure 19. Interdig Bmpr1a inactivation in Bmpr1b null mutants rescues digit one outgrowth.
Figure 19. Interdigit Bmpr1a inactivation in Bmpr1b null mutants rescues digit one outgrowth. Bmpr1b null mice have brachydactyly (C and G). The proximal phalanges of these mutants are malformed and instead a rudimentary phalanx is observed (compare yellow ovals in E and G). This brachydactyly is fully rescued in digit one when Bmpr1a is inactivated in the ID mesenchyme with Osr1-cre in Bmpr1b null mice (compare C and D black oval, and H and G red ovals, I). Planar X-ray photographs were taken of hindlimbs of adult mice and lengths of skeletal elements from three individual mice of each genotype were measured using pixel size (E-H). For quantification of digit rescue, only the proximal phalanges were measured and the most distal normal phalanx was excluded (I). Metatarsal length was similarly quantified (J). In addition to the relevant genotypes that are shown, experimental mice may also contain a copy of Rosa\textsuperscript{mTmG} that was passed on from the mother in A-D.
6.3 Histological examination of digit one rescue in Osr1-Cre; \(Bmpr1a^{\text{flx/null}}; Bmpr1b^{\text{null/null}}\) mutants at P0.5

Throughout endochondral skeletal development of the phalanges the chondrocytes proliferate, differentiate, and ultimately hypertrophy. During this sequential differentiation process, the chondrocytes have distinct morphologies and are located within specific zones of the skeletal element. These morphologically distinct zones can be detected through a histological analysis. At the distal tip of the skeletal element is the resting zone comprised of slowly proliferating periarticular chondrocytes, which have a circular morphology. These cells differentiate into highly proliferating columnar chondrocytes that have an oval and flattened appearance. Next, the columnar chondrocytes differentiate and enlarge into prehypertrophic chondrocytes. Finally, hypertrophic chondrocytes mineralize the surrounding matrix and undergo apoptosis. The remaining cavities provide a matrix for osteoprogenitor cells and bone formation.

To determine whether the rescued proximal phalanx of digit one in Osr1-Cre; \(Bmpr1a^{\text{flx/null}}; Bmpr1b^{\text{null/null}}\) mutants has the appropriate cell types and morphological developmental zones, digit one sections were stained with hematoxylin and eosin (H&E) (Figure 20 A-D). The distinct morphological zones of differentiating chondrocytes flanking the ossified zone in the middle of the phalanx are evident in control H&E sections as well as in sections of the proximal phalanx of Osr1-Cre; \(Bmpr1a^{\text{flx/null}}\) neonates (Figure 20 A and B). In addition, the rudimentary phalanx of \(Bmpr1b\) null littermates is evident (Figure 20C). The presence of an ossified zone and the other morphologically distinct chondrocyte zones in the rescued phalanx of Osr1-Cre;
*Bmpr1a* 

neonates indicate that chondrocyte differentiation is appropriately progressing (Figure 20D). However, the decrease in size of the ossified zone in the rescued phalanx compared to littermate controls suggests that there is a developmental delay in chondrocyte differentiation (compare Figure 20 D to A and B, yellow lines).

The characterization of the Osr1-Cre transgenic line showed that some Cre activity could be observed at the periphery of digit one via β-galactosidase expression (Figure 20 E). One possibility is that the observed rescue is due to the preferential proliferation of the few β-galactosidase positive cells upon *Bmpr1a* inactivation with Osr1-Cre in a *Bmpr1b* null background. Although, this is unlikely since the chondrocyte specific inactivation of both *Bmpr1a* and *Bmpr1b* results in skeletal defects more severe than those observed in BMPR1B null mice and a complete lack of chondrocyte differentiation. Nonetheless, Cre activity was examined via β-galactosidase expression in Osr1-Cre; *Bmpr1a* 

neonates and littermate controls (Figure 20 E-H). Very few blue positive cells were observed throughout the rescued phalanx (Figure 20 H) indicating that the rescue in digit one is not due to the proliferation of Cre positive cells, lacking both *Bmpr1a* and *Bmpr1b*, within the proximal phalanx.
Figure 20. Histological analysis of Osr1-Cre; Bmpr1a<sup>flox/null</sup>; Bmpr1b<sup>null/null</sup> neonates.

Hematoxylin and eosin (H&E) sections of the proximal phalanx of digit one were examined histologically in P0.5 Osr1-Cre; Bmpr1a<sup>flox/null</sup>; Bmpr1b<sup>null/null</sup> neonates and littermate controls (A-D). Similar to littermate controls, distinct morphological zones indicating progression through chondrocyte differentiation can be observed in the rescued phalanx (compare D to A). However, a decrease in size of the zone of ossification is observed (compare D to A, yellow line). In addition, Cre activity throughout the proximal phalanx of digit one was examined via β-galactosidase expression on 20 μm frozen sections of P0.5 neonates (E-H). A similar amount of blue positive cells can be seen throughout the rescued phalanx as in littermate controls (compare H to E). The rudimentary phalanx of Bmpr1b null mutants is marked by a yellow oval (C and G).
6.4 *Gdf5* expression is not rescued in the proximal phalanx of Osr1-Cre; *Bmpr1a*^{flox/null}; *Bmpr1b*^{null/null} embryos

The original characterization of the *Bmpr1b* null mutation determined that although BMPR1B is not essential for the specification of the prechondrogenic lineage it is necessary throughout skeletal development\(^9\). During normal development *Growth Differentiation Factor 5 (Gdf5)* is expressed within the ID mesenchyme starting at approximately E12.0 and begins to be restricted to the presumptive joint interzone at approximately E13.5\(^12\,13\)\(^9\). *Gdf5* is part of the TGFβ superfamily and is necessary for skeletogenesis and joint formation\(^9\,\13\). However, in *Bmpr1b* mutants aberrant *Gdf5* expression is observed throughout the whole digital ray and is strongest at the periphery along the ID mesenchymal boundary (Figure 21C). Furthermore, molecular analysis determined that the expression pattern of *Gdf5* was normal throughout the formation of the condensing mesenchyme and early proliferation, but became aberrant between E13.0 and E13.5\(^9\). Since digit one formation is rescued in Osr1-Cre; *Bmpr1a*^{flox/null}; *Bmpr1b*^{null/null} mice, *Gdf5* expression was examined at E14.5 to determine if the appropriate pattern and level of expression was also restored (Figure 21). Even though a phenotypically normal proximal phalanx is found in Osr1-Cre; *Bmpr1a*^{flox/null}; *Bmpr1b*^{null/null} adults, an abnormal *Gdf5* expression pattern is still observed in digit one at E14.5(Figure 21D). *Gdf5* expression is decreased along the proximal-distal axis of digit one compared to *Bmpr1b* null mutants (compare Figure 21 C and D), but an expression pattern similar to that of control animals is not observed (Figure 21 A and B). It is unclear whether the rescue of skeletogenesis in digit one is independent of GDF5 function, or the observed decrease *Gdf5* in the anterior region of digit one is enough to
rescue digit outgrowth. In addition, a rescue in \textit{Gdf5} could be developmentally delayed
and an examination of the expression pattern at later developmental stages would reveal
if the \textit{Gdf5} expression pattern is rescued by the time digit one is phenotypically normal.
Figure 21. Characterization of Gdf5 expression in Osr1-Cre; Bmpr1aflox/null; Bmpr1bnull/null embryos. Gdf5 expression was examined in E14.5 Osr1-Cre; Bmpr1aflox/null; Bmpr1bnull/null embryos (D) and littermate controls to determine if aberrant expression observed in Bmpr1b mutants (C) was rescued and similar to that of control littermates (A). Although there is a decrease in Gdf5 expression along the proximal-distal axis on the part of digit one (D, ovals), the existing Gfd5 expression is not restricted to the presumptive joint interzone region as observed in control embryos (A and B, ovals).
6.5 Abolishing apoptotic cell death does not rescue brachydactyly in Bmpr1b null mutants

The aberrant Gdf5 expression observed in Bmpr1b null mutants is also associated with excessive apoptotic cell death in the digital rays\textsuperscript{135}. In fact, in a previous analysis of a mouse insertional mutant that lacks Bmpr1b expression, the authors hypothesized that elevated levels of GDF5 signaled through a different type 1 receptor and activated cell death in the developing digital rays\textsuperscript{135}. To test whether cell death causes the Bmpr1b null phenotype, the previously characterized Bak\textsuperscript{null/null}; Bax\textsuperscript{null/null} mutant mice were used to abolish apoptotic cell death\textsuperscript{98,136}. LysoTracker Red analysis of Bak\textsuperscript{null/null}; Bax\textsuperscript{null/null} mutant embryos at various developmental stages (E9.5 – E13.5) showed no detectable cell death while Bak null controls exhibited appropriate levels of LysoTracker Red fluorescence (data not shown). Since cell death occurs during normal development of the joints, skeletal preparations of Bak\textsuperscript{null/null}; Bax\textsuperscript{null/null} E18.5 hindlimbs were first examined to determine if skeletal elements of the autopod developed normally (Figure 22 A and B). Compared to Bak null controls, the Bak; Bax compound null mutants had normal hindlimb development (Figure 22 A and B). This indicates that Bak and Bax are not necessary for normal joint formation to occur during mouse limb development.

To determine if abolishing cell death could rescue brachydactyly of Bmpr1b mutant mice, Bak and Bax were inactivated in a Bmpr1b null background and skeletal preparations of E18.5 hindlimbs were examined (Figure 22 C-E). Abolishing apoptosis in Bmpr1b null mice did not rescue brachydactyly and hindlimbs were still missing proximal phalanges similar to Bmpr1b littermate controls (compare Figure 22 D and E). This indicates that the brachydactylyous phenotype observed in Bmpr1b null mice is not
due to aberrant apoptotic cell death and the rescue of digit one in Osr1-Cre; $Bmpr1a^{\text{flox/null}}; Bmpr1b^{\text{null/null}}$ mutants is not due to the decrease in apoptotic cell death.
Figure 22. Abolishing apoptotic cell death does not rescue brachydactyly in Bmpr1b null mutants. The role of apoptotic cell death was examined during normal skeletal development and in Bmpr1b mutants. Skeletal preparations of E18.5 Baknull/null, Baxnull/null embryos have no observable abnormalities in skeletogenesis or joint formation (A-B). Furthermore, abolishing apoptosis by inactivating Bak and Bax in a Bmpr1b null background did not rescue brachydactyly and phalanx development (C-E).
6.6 Interdigit Bmp7 is necessary for rescue of digit one development in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} mice

Previously published work indicates that BMP signaling from the ID tissue signals to the forming anterior digit and specifies digit identity\textsuperscript{8,41}. For example, implanting beads soaked in Sonic Hedgehog (SHH) protein in chick IDs during development led to transformations in digit identity and an increase in ID Bmp7 expression\textsuperscript{8,41}. However, posteriorization of digit identity was reversed when beads soaked in SHH and NOGGIN, a BMP inhibitor, were simultaneously imbedded in the ID mesenchyme\textsuperscript{8,41}. Conversely, implanting just a NOGGIN soaked bead resulted in anterior transformations of digit identity and a decrease in SMAD 1, 5, and 8 activity within the phalanx forming region\textsuperscript{8,41}. Thus, modulating ID BMP signaling through the regulation of Bmp7 and Noggin can affect digit formation.

Perturbing BMP signaling to the ID mesenchyme through Bmpr1a inactivation in Bmpr1b null mice rescues digit one brachydactyly. Since it has been shown that modulating ID BMP ligands and inhibitors affects digit outgrowth, one possible mechanism is that the lack of ID BMPR1A allows for unbound BMP7 or NOGGIN to diffuse and signal to the developing digit. Furthermore, since BMP7 is an activator of the BMP signaling cascade inactivating ID specific Bmp7 in an Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} background could test whether BMP7 can diffuse from the ID and signal to the developing digit to regulate skeletogenesis. Similarly, inactivation of the BMP inhibitor Noggin in an Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} background can test whether the rescue of brachydactyly in the compound BMP receptor mutants is due to a
decrease in BMP signaling and whether the ID tissue can act as a signaling center during digit development.

*Noggin* null mice, generated by replacing the coding region with the *LacZ* gene, have severe defects in limb development due to hyperplasia and lack of proper joint formation. Because of the severity of the homozygous *Noggin* mutant phenotype, only one copy of the *Noggin* allele was inactivated to test whether decreasing a BMP inhibitor would cause brachydactyly in *Osr1-Cre; Bmpr1a<sup>flox/null</sup>;Bmpr1b<sup>null/null</sup> mutants (Figure 23). Hindlimbs of littermate controls with one copy of *Noggin* inactivated developed normally (N=2) (Figure 23D). Furthermore, the brachydactylous phenotype observed in *Bmpr1b* null mice was not affected with simultaneous inactivation of one copy of *Noggin* (N=2) (Figure 23E). Since inactivating one copy of *Noggin* did not cause digit one brachydactyly in *Osr1-Cre; Bmpr1a<sup>flox/null</sup>;Bmpr1b<sup>null/null</sup> mice (N=2), digit one rescue might not be due to excess inhibition of ID BMP signaling through NOGGIN (Figure 23F).

Similarly, *Bmp7* was inactivated in an *Osr1-Cre; Bmpr1a<sup>flox/null</sup>;Bmpr1b<sup>null/null</sup>* background and skeletal elements of adult hindlimbs were examined (Figure 24). The ID specific inactivation of *Bmp7* as well as ID *Bmp2, 4*, and 7 does not have any apparent effect of limb skeletogenesis (Figure 24D and Figure 18). Although *Bmp7* inactivation did not affect brachydactyly of the phalanges in a *Bmpr1b* null background, shortening of metatarsal one is observed (N=1) (Figure 24E). Finally, inactivating ID *Bmp7* in *Osr1-Cre; Bmpr1a<sup>flox/null</sup>;Bmpr1b<sup>null/null</sup>* mice results in digit one brachydactyly, indicating that ID BMP7 could play a role in digit formation (N=1) (Figure 24F). However, because only one individual with this genotype was assessed, further analysis is necessary to
determine with confidence whether ID specific \( Bmp7 \) inactivation causes brachydactyly in Osr1-Cre; \( Bmp1a^{\text{flox/null}}, Bmp1b^{\text{null/null}} \) mutant mice. Overall, this analysis determines that modulating ID BMP signaling can affect digit outgrowth in certain mutant backgrounds.
Figure 23. Removing one copy of the BMP inhibitor, Noggin, does not affect digit one rescue in Osr1-Cre; Bmpr1a\textsuperscript{floox/null}; Bmpr1b\textsuperscript{null/null} mice.
Figure 23. Removing one copy of the BMP inhibitor, Noggin, does not affect digit one rescue in Osr1-Cre; Bmpr1a$^{\text{flox/null}}$; Bmpr1b$^{\text{null/null}}$ mice. One copy of Noggin was inactivated in Osr1-Cre; Bmpr1a$^{\text{flox/null}}$;Bmpr1b$^{\text{null/null}}$ mice in order to determine if digit one rescue is due to excess NOGGIN upon ID Bmpr1a inactivation. Brachydactyly was not affected in Bmpr1b null mice upon the simultaneous inactivation of Noggin (E). Brachydactyly was not restored in Osr1-Cre; Bmpr1a$^{\text{flox/null}}$; Bmpr1b$^{\text{null/null}}$; Noggin$^{\text{LacZ/wt}}$ (compare F and C, black ovals). Exogenous ossified structures were observed in digits two and three (F, black arrows).
Figure 24. Inactivating ID Bmp7 causes brachydactyly in digit one of Osr1-Cre;

Bmpr1\textsuperscript{a}\textsuperscript{flox/null}; Bmpr1\textsuperscript{b}\textsuperscript{null/null} mice

\begin{center}
\begin{tabular}{ccc}
\textbf{A} & \textbf{B} & \textbf{C} \\
\textbf{D} & \textbf{E} & \textbf{F} \\
\end{tabular}
\end{center}
Figure 24. Inactivating ID Bmp7 causes brachydactyly in digit one of Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} mice. ID Bmp7 was inactivated in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} mice in order to determine if digit one rescue is due to available ID BMP7 after ID Bmpr1a inactivation. Brachydactyly of the phalanges was not affected in Bmpr1b null mice upon the simultaneous inactivation of ID Bmp7, however metatarsal one appears to be shorter compared to Bmpr1b null littermate controls (compare B and E). Furthermore, digit one brachydactyly was restored in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null}; Bmp7\textsuperscript{flox/null} hindlimbs (compare F and C, black ovals).
6.7 Summary

Inactivation of ID Bmp2, 4, and 7 with Osr1-Cre did not affect skeletogenesis or digit identity. However, inactivation of ID Bmpr1a with Osr1-Cre in a Bmpr1b null background completely rescues brachydactyly in the proximal phalanx of digit one. Histological analysis of Osr1-Cre; Bmpr1a\textsuperscript{flox/null}, Bmpr1b\textsuperscript{null/null} neonates determined that chondrocyte proliferation and differentiation during skeletogenesis occurs appropriately, but possibly with a developmental delay. In addition, although Gdf5 within digit one appears slightly lower compared to Bmpr1b null littermates, the Gdf5 expression pattern is still abnormal. Furthermore, brachydactyly was not rescued in Bak\textsuperscript{null/null}; Bax\textsuperscript{null/null}; Bmpr1b\textsuperscript{null/null} mutants with abolished apoptosis. Finally, the effects on digit one rescue were assessed upon modulation of ID BMP signaling in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} mutants. Inactivating one copy of Noggin did not affect brachydactyly in Bmpr1b null mice or affect digit one rescue in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} mutants. Conversely, inactivating ID Bmp7 in an Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} background recapitulated the brachydactyloous phenotype observed in Bmpr1b null mice.

6.8 Discussion

Bmpr1b null mice have been previously characterized and have brachydactyly\textsuperscript{91,135}. Most notably, the proximal phalanx in digit one and the proximal and middle phalanges in digits two through five are severely malformed with a rudimentary skeletal element\textsuperscript{91,135}. This skeletal defect in adults is associated with aberrant Gdf5 expression and excessive cell death during development\textsuperscript{91,135}. However, abolishing apoptosis in Bmpr1b null mice did not affect brachydactyly indicating that the previously
observed aberrant cell death could be a secondary phenotype. However, the normal joint formation in Bak\textsuperscript{null/null}; Bax\textsuperscript{null/null} mutants suggests that an alternative cell death pathway could be activated in the absence of apoptosis\textsuperscript{118,136}.

Inactivating ID Bmpr1a in a Bmpr1b null background completely rescued outgrowth of the proximal phalanx as well as chondrocyte proliferation and differentiation during skeletogenesis. However, an aberrant Gdf5 expression pattern was still observed in the rescued digital ray during embryonic development. This suggest that skeletogenesis is rescued through a Gdf5 independent mechanism.

It has been demonstrated that modulating ID BMP signaling though NOGGIN soaked beads or an increase in Bmp7 expression affects digit outgrowth and identity during chick development\textsuperscript{8,41}. Therefore, digit one rescue in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} could occur through BMP ligand or inhibitor secretion from the ID mesenchyme. To test whether BMP ligand secretion from the ID region regulates digit formation ID Bmp2, 4, 7 were inactivated with Osr1-Cre at E13.5. Skeletal preparations of Bmp2, 4, and 7 mutant hindlimbs appeared completely normal, indicating that during normal development ID BMP2, 4, and 7 secretion at approximately the time of PCD initiation does not affect skeletogenesis.

Even though inactivation of ID ligands did not affect skeletogenesis, during normal development ID BMP ligands would be binding to BMPR1A. Therefore, the presence of BMPR1A could be confining BMP ligands to the ID mesenchyme. Consequently, in the absence of ID BMPR1A, BMP ligands (or inhibitors) are no longer sequestered and can diffuse and signal to the developing digits. In fact, preliminary evidence suggests that inactivation of ID Bmp7 in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null}
mice produces the brachydactylos phenotype observed in \textit{Bmpr1b} null mutants. This indicates that ID BMP signaling could affect skeletogenesis. Nonetheless, further analysis is necessary to fully characterize how ID \textit{Bmp7} can affect outgrowth and skeletogenesis in the absence of \textit{Bmpr1b} throughout the developing phalanx.
Limb development provides a rich context to study how complex information from different tissue types and signaling pathways is appropriately integrated to regulate processes such as programmed cell death and morphogenesis during normal development. Past attempts to determine whether BMP signaling directly regulates PCD could not distinguish a direct role from an indirect role through AER-FGF regulation\textsuperscript{42,43,48,57,70,73}. Utilizing the Cre-LoxP recombination system\textsuperscript{12} to manipulate BMP gene expression selectively within the ID mesenchyme, a direct role for BMP signaling in the regulation of PCD was established. Tissue-specific manipulation of the ID mesenchyme also provided evidence that BMP signaling from the ID tissue influences digit outgrowth and skeletogenesis.

The modulation of ID BMP signaling was accomplished through two different approaches – the inactivation of BMP ligands to perturb signaling \textit{from} the ID mesenchyme and the inactivation of BMP receptors to perturb signaling \textit{to} the ID mesenchyme. In both of these manipulations, mutants have a decrease in ID apoptosis during embryonic development and syndactyly, without an effect on AER-FGF and retinoic acid signaling. However, a distinct difference in the areas of observed decrease in cell death and syndactyly exists between the ligand and receptor mutants. In the case of ID \textit{Bmpr1a} inactivation, syndactyly is observed in ID1 through ID3. In the ID \textit{Bmp2, 4,} and 7 ligand inactivation, webbing is observed only in ID2 and ID3. Thus, a subset of the \textit{Bmpr1a} syndactylous phenotype is observed when \textit{Bmp2, 4,} and 7 are inactivated in the ID mesenchyme. This could be attributed to the variability observed in Osr1-Cre recombination. With incomplete ID \textit{Bmp} ligand inactivation, secretion of the remaining
ligand from the ID mesenchyme could signal to and initiate the BMP pathway throughout the whole ID and result in a less severe phenotype. However, upon cell autonomous \( Bmpr1a \) inactivation, BMP signaling through receptor activation will be completely abolished in Cre expressing ID cells regardless of the variability in Cre expression throughout the ID region. An alternative explanation for the difference in severity of syndactyly between the receptor and ligand inactivation can be attributed to differences in half-life of the Bmp ligand and receptor mRNAs and/or protein products. Finally, although inactivation of the ID \( Bmp2, 4, \) and 7 ligands in a genetic series indicated that \( Bmp7 \) is necessary for ID PCD and \( Bmp2 \) and 4 are redundant, the more severe syndactyly in \( Bmpr1a \) mutants could be due to an additional ligand regulating ID PCD through BMPR1A during normal development. BMP5 is one such candidate that is expressed in the ID mesenchyme and has previously been implicated in ID PCD regulation during chick limb development\(^{50}\).

Characterization of Osr1-Cre; \( Bmpr1a^{flox/null} \) embryos suggests that the direct and cell autonomous BMP regulation of ID PCD may be through both a SMAD dependent and SMAD independent mechanism. Analysis of mutants with inactivated ID \( Bmp2, 4, \) and 7 revealed a decrease in pSMAD 1 and 5 within IDs with decreased PCD, while upon ID \( Bmpr1a \) inactivation some pSMAD was still observed in the proximal ID regions associated with the greatest decrease in PCD. Since there are multiple BMP type 1 receptors in the ID mesenchyme, inactivation of the ligands likely perturbs SMAD signaling cascades independent of the process of PCD as well as the non-canonical BMP regulation of PCD through BMPR1A. Previous studies characterizing the role of BMP7 in nephron development demonstrated that signal transduction occurred through a SMAD
independent cascade involving p38 phosphorylation\textsuperscript{95}. Further analysis of ID \textit{Bmp}2, 4, and 7 mutants is necessary to determine whether p38 phosphorylation or an alternate, SMAD independent, a pathway is necessary for PCD regulation.

\textit{In vitro} experiments utilizing limb cultures could elucidate which SMAD independent BMP pathways necessary for PCD regulation are activated through BMPR1A. Limb culture models utilizing small molecule inhibitors to study the regulation of ID PCD have been previously established\textsuperscript{43,50}. To test whether a p38 dependent pathway is involved in ID PCD, wildtype E13.5 limb cultures treated with the small molecule inhibitor SB 203580 could be assessed for changes in total cell death as well as AER-\textit{Fgf}8 and \textit{Rarβ} expression. A decrease in cell death with no change in AER-FGF and retinoic acid levels in limbs cultured with SB 203580 would suggest that p38 phosphorilation is necessary for ID PCD, likely through ID BMP signaling. While genetic manipulations have determined that \textit{Bmpr1a} is necessary for PCD during normal development, such an \textit{in vitro} analysis would elucidate the post-translational interactions necessary for SMAD-independent BMP regulation of PCD.

Although the ID mesenchyme is ultimately removed through PCD allowing for complete digit separation, it has been implicated to act as a signaling center necessary for proper digit outgrowth\textsuperscript{8,41,85}. Hindlimbs of mice with ID \textit{Bmp}2, 4, and 7 inactivated with \textit{Osr1-Cre} formed normal skeletal elements, indicating that the secretion of BMP ligands from the ID mesenchyme at ~ E13.5 is not necessary for digit development. However, the differentiation of the limb bud mesenchyme into digital ray condensations separated by ID tissue has already occurred by ~E12.0. It is therefore possible that a required signal from the ID has completed its patterning role by the time \textit{Osr1-Cre} activity is observed at
E13.5\textsuperscript{36,37}. Currently, the genetic tools for ID specific inactivation at ~ E12.0 – E12.5 are not available to address this possibility. Nonetheless, the rescue of digit one brachydactyly upon ID Bmpr1a inactivation in Bmpr1b null mice demonstrates that the ID mesenchyme has the potential to act as a signaling center for digit outgrowth.

Preliminary evidence that the concomitant inactivation of ID Bmp7 results in the brachydactylyous phenotype supports the idea that appropriate levels of BMP signaling from the ID can play a role in digit identity\textsuperscript{8,41}. In order to determine whether changes in BMP signaling within digit one rescue brachydactyly in Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} mice, further characterization of post-translational signal transduction components, such as SMAD phosphorylation in the rescued phalanx, is necessary.

Furthermore, transcriptome and microproteomic analyses of E13.5 ID tissue comparing Osr1-Cre; Bmpr1a\textsuperscript{flox/null}; Bmpr1b\textsuperscript{null/null} to Bmpr1b null littermate controls could elucidate novel factors within the ID mesenchyme that signal to the developing digits.

Overall this work has provided novel insight into the role of ID BMP mesenchymal signaling during normal limb development (Figure 25). Although the ID is ultimately removed through BMP regulated apoptosis, it can also provide cues to the forming digits that are necessary for normal outgrowth and development. Furthermore, my research demonstrates that BMPs are direct effectors of ID apoptosis and, in coordination with FGF and retinoic acid signaling, are necessary for PCD. In particular, it was established that BMP7 is necessary for ID PCD while BMP2 and 4 act redundantly. Furthermore, these ID ligands likely signal through BMPR1A, which is necessary for the cell autonomous regulation of ID apoptosis, and BMPR1B, which plays a redundant role in ID PCD. Much is still unknown about how signal transduction from
the FGF, BMP, and retinoic acid pathways could be integrated. Thus, given the prevalence of congenital limb defects, future characterization of how these pathways interact in the process of cell death regulation during normal development is necessary to further elucidate what goes awry in many pathogenic states.
Figure 25. Updated model of interdigit programmed cell death regulation during normal development
Figure 25. Updated model of interdigit programmed cell death regulation during normal development. Multiple pathways have been implicated in the regulation of ID PCD. In the current model, PCD is thought to be regulated by an antagonistic relationship between FGF secretion from the Apical Ectodermal Ridge and Retinoic Acid metabolism in the proximal region of the ID mesenchyme. This schematic demonstrates how FGF signaling acts as a cell survival factor by inhibiting PCD, while the retinoic acid pathway activates cell death signaling cascades. In addition, this thesis work has provided genetic evidence that BMP signaling directly regulates ID PCD through a reactive oxygen species (ROS) mediated mechanism and can affect digit development. In particular, ID BMP7 secretion is necessary for PCD while BMP2 and BMP4 are redundant. These ID BMP ligands likely signal through BMPR1A and to a lesser degree BMPR1B. ID BMP signaling through BMPR1A is necessary for PCD during normal development, while BMPR1B plays a redundant role in PCD regulation. ID, interdigit.
APPENDIX: Examining interdigit transcriptome changes in the Msx2-Cre; 

*Bmpr1a<sub>flox/null</sub>* syndactylous mutant

BMPs’ indirect regulation of ID programmed cell death was determined through the tissue-specific Msx2-Cre; *Bmpr1a* inactivation in the apical ectodermal ridge<sup>42</sup>. These mutants have a decrease in total ID PCD during embryonic development that leads to syndactyly in adults<sup>42</sup>. It was determined that the decrease in cell death is due to the spatio-temporal increase in *Fgf4* and *Fgf8* expression from the AER<sup>42</sup>. This increase in AER-FGF secretion and signaling to the underlying mesenchyme inhibits the process of PCD without affecting ID BMP signaling<sup>42</sup>. To determine transcriptome changes in the ID mesenchyme a microarray analysis approach was used. To accomplish this, the first ID tissue (the most anterior ID) was dissected from the left and right forelimbs of mutants and controls (*Bmpr1a<sub>flox/wt</sub>* as controls and Msx2-Cre; *Bmpr1a<sub>flox/null</sub>* as mutants). RNA was extracted from a pool of four IDs (two embryos) and quality and quantity of the extracted RNA was evaluated using an Agilent bioanalyzer and Pico detection kit. The Affymetrix Mouse Genome 430 2.0 Array platform was used and four arrays were used for each genotype. For each array RNA extracted from 6 embryos with a RIN number of equal to or greater than 7.9 was used. Finally, a list of genes with a statistical significant difference in expression was generated with at least a two-fold change in expression and a parametric p-value ≤0.05 (*Figure 26 and 27*). Note that even though the decrease in ID PCD of Msx2-Cre; *Bmpr1a<sub>flox/null</sub>* mutants is due to AER-*Fgf* upregulation, changes in BMP related genes are observed in the microarray analysis (*Figure 26 and 27, red boxes*). This provides further evidence that ID PCD is likely regulated through the integration of signal transduction from multiple signaling pathways (*Figure 25*).
Figure 26. Transcriptome analysis of genes that are at least two-fold upregulated in ID1 of Msx2-Cre; Bmpr1a^{flox/null} E13.5 embryos
Figure 26. Transcriptome analysis of genes that are at least two-fold upregulated in ID1 of Msx2-Cre; Bmpr1a^{fl/nu} E13.5 embryos. To examine which genes are upregulated in ID1 of Msx2-Cre; Bmpr1a^{fl/nu} E13.5 embryos, statistical analysis was performed on probe-intensity level data (CEL files) using GeneSpring GX software. Genes were considered statistically significant if at least a two-fold change in expression with a parametric p-value $\leq$ 0.05 was determined. Note a change in ID Bmp5 expression, a gene previously implicated in PCD regulation (B, red box)\textsuperscript{50}. FC – fold change, Reg – regulation.
Figure 27. Transcriptome analysis of genes that are at least two-fold downregulated in ID1 of Msx2-Cre; Bmpr1a<sup>fl<sub>ox</sub>/null</sup> E13.5 embryos. To examine which genes are downregulated in ID1 of Msx2-Cre; Bmpr1a<sup>fl<sub>ox</sub>/null</sup> E13.5 embryos, statistical analysis was performed on probe-intensity level data (CEL files) using GeneSpring GX software. Genes were considered statistically significant if at least a two-fold change in expression with a parametric p-value ≤0.05 was determined. Note a change in ID Bmpr expression, a secreted BMP inhibitor (B, red box)<sup>141</sup>. FC – fold change.
REFERENCES


Benazet, J. D. et al. Smad4 is required to induce digit ray primordia and to initiate the aggregation and differentiation of chondrogenic progenitors in mouse limb buds. Development 139, 4250-4260, doi:10.1242/dev.084822 (2012).


CURRICULUM VITAE

Date and Location of Birth: March 30th, 1986 in Sofia, Bulgaria

Contact Information:
Candidate: Maria Mateeva Kaltcheva
Address: N5563 Redwing Dr., Fond du Lac, WI 54937
Mobile: 414-364-6573
Email: kaltchevam@gmail.com, kaltchevam@mail.nih.gov, mkaltch1@jhu.edu

Education:
2008 – 2015 Ph.D. in Biology (with a focus on Developmental Biology) Johns Hopkins University (JHU), Baltimore, MD and the National Institutes of Health (NIH), Bethesda, MD Graduate Partnership Program (GPP)
2008 B.S. (Biochemistry with Honors), University of Wisconsin, Madison, Wisconsin

Brief Chronology of Research Employment:
2008 – present Pre-doctoral CRTA Fellow, Cancer and Developmental Biology Laboratory, Center for Cancer Research, National Cancer Institute, National Institutes of Health–Johns Hopkins University Graduate Partnership Program
2006 – 2008 Undergraduate Research, Laboratory of Dr. Atwood, University of Wisconsin – Madison
• Utilized human embryonic stem cell culture to investigate the roles of tau and GSK3-beta during stem cell differentiation in order to better understand the developmental role of the proteins implicated in Alzheimer’s.
• Became proficient in human embryonic stem cell culture and differentiation (neuroectodermal) techniques and maintenance of mouse embryonic fibroblasts.
2005 – 2006 Undergraduate Research, Laboratory of Dr. Sutula, University of Wisconsin – Madison
• Certified to work with rats in a laboratory setting and perform kindling to stimulate epileptic seizures
2005 Undergraduate Research, Laboratory of Dr. Geissinger, University of Wisconsin – Milwaukee
• Worked on developing new fiber optic cladding materials with biological sensors

Grants and Awards:

2015  Outstanding Poster, 2015 NCI – Frederick Spring Research Festival

2014  Outstanding Poster, 2014 NCI – Frederick Spring Research Festival

2013  Outstanding graduate student talk, runner up, 2013 Society for Developmental Biology Mid – Atlantic Regional Meeting

2012  Winner of the NIH Intramural Fellows Award for Research Excellence (FARE) 2013 competition

2012  Outstanding Poster, 2012 NCI – Frederick Spring Research Festival

2011  Outstanding Poster, 2011 NCI – Frederick Spring Research Festival

2007  Hilldale Undergraduate/Faculty Research Fellowship Scholarship, University of Wisconsin – Madison

2005  Best Undergraduate Poster Presentation, Laboratory for Surface Studies Summer Student Symposium, University of Wisconsin - Milwaukee

Teaching and Mentoring Experience

2009  Teaching Assistant, Cell Biology Laboratory, JHU

2008  Teaching Assistant, Biochemistry Laboratory, JHU

Leadership Positions:

2012  Co-Chair of the Graduate Student Council of the NIH Graduate Partnership Program

2011  Johns Hopkins University – Graduate Partnership Program Class Representative

2008  JHU Biology Graduate class representative

2006 – 2007  Officer/Technical chair of the UW – Madison Undergraduate
Neuroscience Society

Member of the Golden Key Honors Society – UW-Madison chapter

**Professional Societies:**

2010 – Present  Society for Developmental Biology

**Editorial Reviews:**

Reviewed manuscripts for:
- PLoS Genetics
- Development, Growth and Differentiation

**Presented Research**


4. Maria M Kaltcheva, Brian Harfe and Mark Lewandoski. (Poster) Interdigital BMP signaling is essential for programmed cell death. CSH Stem Cell Biology meeting September 2013.

5. Maria M Kaltcheva, Sangeeta Pajni-Underwood, Brian Harfe and Mark Lewandoski. (Talk) Interdigital Bone Morphogenetic Protein Signaling is Essential for Programmed Cell Death and is implicated in Digit Formation. Society for Developmental Biology Mid-Atlantic Regional Meeting April 2013.

6. Maria M Kaltcheva, Sangeeta Pajni-Underwood, Brian Harfe and Mark Lewandoski. (Poster) Interdigital Bone Morphogenetic Protein Signaling is Essential for Programmed Cell Death and is implicated in Digit Formation. NCI – Frederick Spring Research Festival May 2013.
7. Maria M Kaltcheva, Sangeeta Pajni-Underwood, Brian Harfe and Mark Lewandoski. (Poster) Interdigit Bone Morphogenetic Protein Signaling is Essential for Programmed Cell Death and is implicated in Digit Formation. NIH Research Festival October 2012.

8. Maria M Kaltcheva, Sangeeta Pajni-Underwood, Brian Harfe and Mark Lewandoski. (Poster) Interdigit Bone Morphogenetic Protein Signaling is Essential for Programmed Cell Death and is implicated in Digit Formation. 12th International Conference on Limb Development and Regeneration 2012.


