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The ERK MAPK Pathway
in Bone and Cartilage Formation

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1. Introduction

The Extracellular signal-related kinase (ERK) Mitogen-activated protein kinase (MAPK) pathway is a signaling cascade that is activated by various extracellular stimuli including fibroblast growth factors. The ERK MAPK pathway has recently been shown to play critical roles in skeletal development. A number of human skeletal syndromes have been shown to result from mutations in this pathway. These include Noonan, Costello, and cardio-facio-cutaneous syndromes (Aoki et al., 2005; Pandit et al., 2007; Rodriguez-Viciana et al., 2006). In addition, activating mutations in FGFR2 cause craniosynostosis syndromes such as Apert and Crouzon syndromes, while activating mutations in fibroblast growth factor receptor 3 (FGFR3) are responsible for the most common forms of human dwarfism, achondroplasia, thanatophoric dysplasia, and hypochondroplasia (Bellus et al., 1995; Jabs et al., 1994; Rousseau et al., 1994, 1995; Rutland et al., 1995; Shiang et al., 1994; Wilcox et al., 1998; Wilkie et al., 1995).

Although a number of in vitro experiments indicated profound effects of the ERK MAPK pathway on chondrocyte and osteoblast phenotype, sometimes conflicting results were reported presumably due to variable culture conditions (Bobick & Kulyk, 2008; Schindeler & Little, 2006), and the roles of the ERK MAPK pathway in vivo remained elusive. Therefore, to examine the role of ERK MAPK in skeletal development, we used both gain-of-function and loss-of-function approaches to activate or inactivate the ERK MAPK pathway in skeletal tissues of genetically engineered mice. We used the Cre-*loxP* system to inactivate *ERK1* and *ERK2* in skeletal tissues (Logan et al., 2002; Matsushita et al., 2009a). By using the *Prx1-Cre* transgene, mice lacking *ERK1* and *ERK2* in the limb and head mesenchyme were created. We also generated a loss of function model in chondrocytes by using the *Col2a1-Cre* transgene (Matsushita et al., 2009a; Ovchinnikov et al., 2000). To induce postnatal inactivation of *ERK1* and *ERK2* in chondrocytes, we used the *Col2a1-CreER* transgene to express a tamoxifen-inducible form of Cre recombinase (Nakamura et al., 2006; Sebastian et al., 2011). For gain-of-function experiments, we generated *Prx1-MEK1* transgenic mice that express a constitutively active mutant of MEK1 in undifferentiated mesenchymal cells under the control of a *Prx1* promoter (Matsushita et al., 2009a). We also generated *Col2a1-MEK1* transgenic mice that express a constitutively active mutant of MEK1 in chondrocytes under the control of the regulatory sequences of *Col2a1* (Murakami et al., 2004). In this review, we will summarize the roles of the ERK MAPK pathway in skeletal development based on our recent studies using genetically engineered mouse models.

2. Skeletal development and ossification processes

Bone formation takes place in two major ossification processes, endochondral ossification and intramembranous ossification (Colnot, 2005; Hunziker, 1994; Opperman, 2000; Shapiro et al., 2005). Both chondrocytes and osteoblasts arise from common undifferentiated mesenchymal progenitor cells. In endochondral ossification, the skeletal element is formed as a cartilaginous template that is subsequently replaced by bone. Undifferentiated mesenchymal cells first aggregate to form mesenchymal condensation and differentiate into chondrocytes. Chondrocytes proliferate in columnar stacks to form the growth plate, then exit the cell cycle, and differentiate into hypertrophic chondrocytes. The cartilaginous matrix of hypertrophic chondrocytes is calcified and subsequently invaded by blood vessels. Hypertrophic chondrocytes are removed by apoptotic cell death, and the cartilaginous matrix is resorbed by chondroclasts/osteoclasts and replaced by trabecular bone. Chondroclast/osteoclast formation is supported by receptor activator of nuclear factor-kappa B ligand (RANKL) secreted from osteoblasts and bone marrow stromal cells (Kim et al., 2000; Yasuda et al., 1998). In intramembranous ossification, mesenchymal cells directly differentiate into bone-forming osteoblasts; cortical bone is formed by osteoblasts that arise from the osteochondro progenitor cells in the perichondrium. The entire process of endochondral ossification and intramembranous ossification is under the control of various hormones and growth factors. These include systemic factors such as growth hormone, estrogen, and glucocorticoids; and local factors such as Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTHrP), fibroblast growth factors (FGF), transforming growth factor-β (TGF-β), and bone morphogenetic proteins (BMP) (DeLise et al., 2000; van der Eerden et al., 2003).

3. The ERK MAPK pathway and human syndromes

3.1 The ERK MAPK pathway

The ERK MAPK pathway (Fig. 1), which is activated by various stimuli in eukaryotic cells, transduces extracellular signals into cells and coordinates cellular responses. The MAPK pathways are generally organized into three kinase modules: MAPKK kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK. A diverse array of growth factors and cytokines transduce their signals through the activation of the small G protein Ras, which leads to the activation of the Raf members of MAPKKK, and then to the activation of MAPKK, MEK1 and MEK2. MEK1 and MEK2 then phosphorylate and activate MAPK, ERK1 and ERK2. ERK1 and ERK2 then phosphorylate various cytoplasmic and nuclear target proteins, ranging from cytoplasmic adaptor proteins and transcription factors to kinases including RSK (Cargnello & Roux, 2011; Roux & Blenis, 2004). The ERK MAPK pathway has been shown to mediate the intracellular signaling induced by a variety of growth factors such as FGFs, BMPs, and TGFs (Jun et al., 2010; Mu et al., 2011; Murakami et al., 2000; Osyczka & Leboy, 2005; Qureshi et al., 2005; Tuli et al., 2003).

3.2 Human syndromes caused by mutations in the MAPK pathway

Recently, a number of human mutations have been identified in the molecules in the MAPK cascade (Fig. 1). Missense activating mutations in KRAS, BRAF, MEK1, and MEK2 have

been identified in Cardio-facio-cutaneous syndrome (Rodriguez-Viciana et al., 2006). KRAS mutations have been also identified in Noonan syndrome (Schubbert et al., 2006). HRAS mutations cause Costello syndrome (Aoki et al., 2005), and loss-of-function mutations in RSK2 cause Coffin-Lowry syndrome (Trivier et al., 1996). In addition, haploinsufficient expression of ERK2 has been associated with DiGeorge syndrome (Newbern et al., 2008). All of these syndromes present with various skeletal manifestations, including short stature and craniofacial and limb abnormalities, underscoring the importance of the MAPK pathway in human skeletal development (Hanauer & Young, 2002; Hennekam, 2003; Noonan, 2006; Reynolds et al., 1986; van der Burgt, 2007).
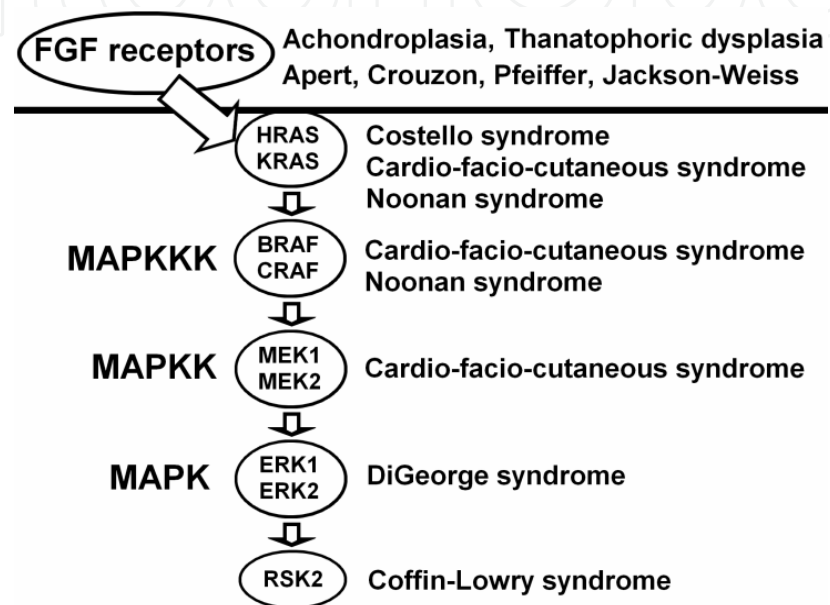

Fig. 1. The ERK MAPK pathway and human skeletal syndromes

4. Genetic manipulation of molecules in the ERK MAPK pathway in mice

To examine the role of the MAPK pathway, various mutant mice have been generated. Inactivation of Rsk2, a downstream kinase of the MAPK pathway, caused a widening of cranial sutures at birth, similar to the delayed closure of fontanelles in patients with Coffin-Lowry syndrome (David et al., 2005; Yang et al., 2004). These observations indicate that Rsk2 plays a critical role in osteoblast differentiation. In contrast to *Rsk2*-null mice, the inactivation of ERK, MEK, and Raf family members has provided little information regarding skeletal development. *ERK1*-null mice are viable and fertile and develop normally without obvious skeletal abnormalities, suggesting that ERK1 is dispensable for skeletal development (Pages et al., 1999; Selcher et al., 2001). In remarkable contrast, *ERK2*-null mice show early embryonic lethality at E6.5, precluding the analysis of skeletal development (Saba-El-Leil et al., 2003; Yao et al., 2003). *Mek1*-null embryos die at E10.5 due to placental defects, while *Mek2*-null mice develop normally without any obvious abnormalities (Belanger et al., 2003; Giroux et al., 1999). *Araf*-null mice show neurological and gastrointestinal defects, but do not show an obvious skeletal phenotype (Pritchard et al., 1996). *Braf*-deficient embryos die at midgestation due to vascular defects, precluding the analysis of skeletal development (Wojnowski et al., 1997). *Craf*-null mice show placental defects and die at around E10.5-12.5 on the C57BL/6 and 129 backgrounds. On the outbred

CD1 background, two-thirds of embryos reach term and die soon after birth. These surviving embryos show a mild delay in ossification; however, it is not clear whether the observed skeletal phenotype is primarily caused by *Craf* deficiency in the skeletal tissues (Wojnowski et al., 1998). Furthermore, mice with chondrocytes deficient in both *Araf* and *Braf* showed normal endochondral bone development (Provot et al., 2008). These observations suggest that members of the ERK, MEK, and Raf family are functionally redundant, while some of the tissue-specific functions are not fully compensated by other family members. To circumvent early embryonic lethality caused by the systemic inactivation of the target gene, tissue-specific inactivation would be essential. Furthermore, the inactivation of multiple family members may be necessary to uncover the roles of ERK, MEK, and Raf family members in skeletal development.

5. Inactivation of ERK1 and ERK2 in undifferentiated mesenchymal cells disrupts bone formation and induces ectopic cartilage formation

Since early embryonic lethality hampered researchers from analyzing the role of the ERK MAPK pathway in skeletal development in vivo as described above, we used the Cre-*loxP* system to inactivate *ERK1* and *ERK2* in skeletal tissues. We used *Prx1-Cre* transgenic mice that express Cre recombinase under the control of the 2.4 kb *Prx1* promoter (Logan et al., 2002) to inactivate *ERK1* and *ERK2* in the limb and head mesenchyme (Matsushita et al., 2009a). The *Prx1* promoter has been shown to direct transgene expression in undifferentiated mesenchyme in the developing limb buds and head mesenchyme. The transgene expression is detectable as early as E10.5, and the transgene expression is confined to the periosteum of the long bones and tendons of the limbs at E15.5 (Logan et al., 2002). We analyzed *ERK1*-null mice and *ERK2*flox/flox; *Prx1-Cre* mice, and these mice did not show obvious skeletal abnormalities. Therefore, we further inactivated *ERK2* in the *ERK1*-null background to totally inactivate *ERK1* and *ERK2* in the head and limb mesenchyme of mouse embryos using the *Prx1-Cre* transgene (Matsushita et al., 2009a).

5.1 Inactivation of ERK1 and ERK2 in mesenchymal cells disrupts osteoblast differentiation

Skeletal preparation of *ERK1* -/-; *ERK2* flox/flox; *Prx1-Cre* mutants revealed severe limb deformities as well as calvaria defects characterized by delayed closure of the cranial sutures (Fig. 2). Histological analysis of the long bones showed disruption of bone formation. These findings indicate that ERK1 and ERK2 play an essential role in bone formation. In situ hybridization analysis indicated that master osteogenic transcription factors *Runx2*, *Osterix*, and *Atf4* were expressed at normal levels, while expression of *Osteocalcin*, a marker of mature osteoblasts, was strongly decreased in *ERK1* -/-; *ERK2* flox/flox; *Prx1-Cre* mice. These observations suggest that osteoblast differentiation was blocked after *Runx2, Osterix,* and *Atf4* expression and before *Osteocalcin* expression. The impaired bone formation was associated with decreased beta-catenin protein levels in the periosteum, suggesting decreased Wnt signaling. We also found that other transcriptional regulators such as *Krox20, Fra1, Fra2, cFos*, and *Cbfb* were downregulated in *ERK1* -/-; *ERK2* flox/flox; *Prx1-Cre* embryos. While the regulatory mechanisms of osteoblast differentiation require further investigation, ERK1 and ERK2 are likely to control skeletal development and osteoblast differentiation through multiple downstream molecules. Consistent with our observation, Ge et al. reported

that transgenic mice that express dominant negative MEK1 under an *Osteocalcin* promoter showed delayed bone formation and reduced mineralization of calvaria, while mice that express a constitutively active MEK1 under the *Osteocalcin* promoter showed accelerated bone formation (Ge et al., 2007). These observations also indicated a critical role of ERK1 and ERK2 in osteoblast differentiation. In vitro studies have also suggested that the ERK MAPK pathway regulates osteoblast differentiation through phosphorylation and acetylation of Runx2 (Ge et al., 2007; Park et al., 2010; Xiao et al., 2000, 2002). The importance of the ERK MAPK pathway in osteoblast differentiation in head mesenchyme was also demonstrated by Shukla et al., who showed that craniosynostosis in a mouse model of Apert syndrome that carries a mutation in *Fgfr2* was prevented by the treatment of MEK1/2 inhibitor (Shukla et al., 2007). These observations further link the activation of the ERK MAPK pathway to the pathogenesis of craniosynostosis syndromes caused by activating mutations in FGFR2.
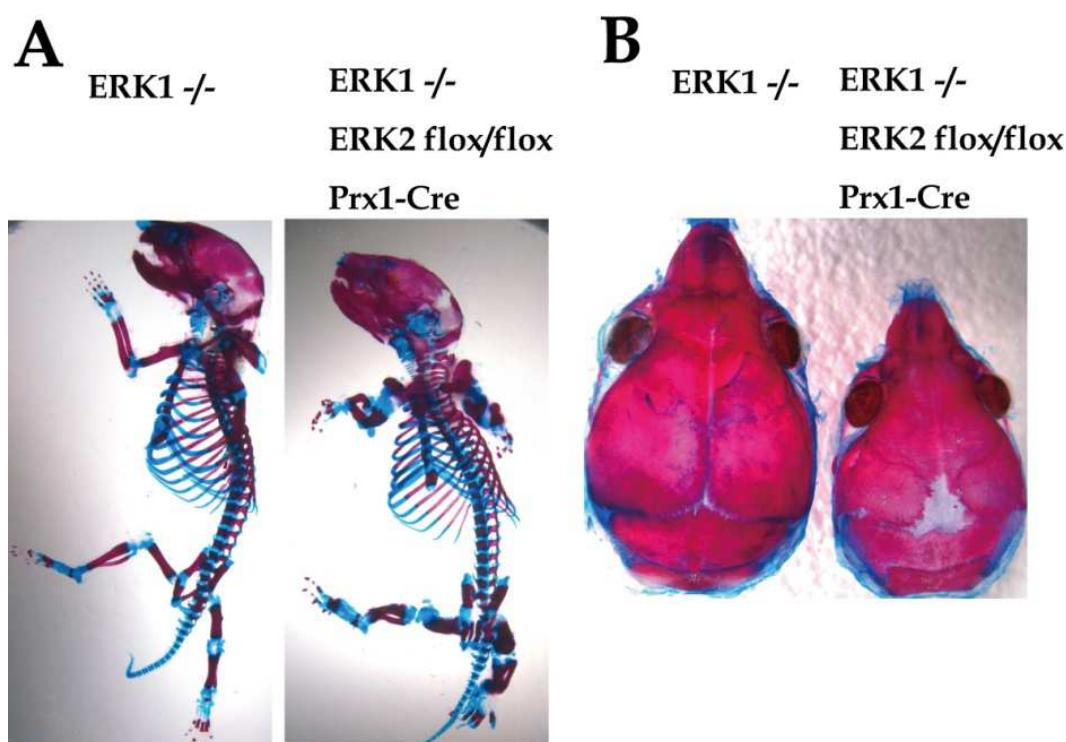

Fig. 2. (A) Skeletal preparation after alizarin red and alcian blue staining at postnatal day 1. (B) Skeletal preparation after alizarin red and alcian blue staining at postnatal day 5.

5.2 Inactivation of ERK1 and ERK2 disrupts osteocyte differentiation

Osteoblasts undergo sequential steps of differentiation and subsequently become embedded in bone matrix as osteocytes. Osteocytes function as a mechanosensor in the bone and secrete dentin matrix protein 1 (Dmp1) and FGF23 to regulate phosphate homeostasis (Dallas & Bonewald, 2010; Feng et al., 2006; Tatsumi et al., 2007; Xiao and Quarles, 2010). Although *ERK1-/-*; *ERK2flox/flox*; *Prx1-Cre* mice showed a remarkable impairment of bone formation, bone-like architecture was observed in the diaphyses of long bones, and osteocyte-like cells were found within the bone-like matrix. Our real-time PCR and immunohistochemical analysis indicated a strong decrease in Dmp1 expression in the skeletal elements of *ERK1-/-*; *ERK2 flox/flox*; *Prx1-Cre* mice. Furthermore, scanning electron

microscopic analysis revealed that osteocytes in *ERK1-/-*; *ERK2flox/flox*; *Prx1-Cre* mice lack dendritic processes, indicating that *ERK1* and *ERK2* inactivation disrupts the formation of osteocyte-lacunar-canalicular system (Kyono et al., 2011). These observations indicate that ERK signaling is essential for *Dmp1* expression and osteocyte differentiation.

5.3 Inactivation of ERK1 and ERK2 in mesenchyme causes ectopic cartilage formation

While inactivation of *ERK1* and *ERK2* inhibited osteoblast differentiation and bone formation, we found ectopic cartilage formation in the perichondrium of *ERK1 -/-; ERK2 flox/flox; Prx1-Cre* mice (Fig. 3). The ectopic cartilage expressed *Sox9*, a transcription factor for chondrocyte differentiation, and *Col2a1*, the gene for type II collagen (Matsushita et al., 2009a). These findings suggest that inactivation of *ERK1* and *ERK2* in mesenchyme inhibited osteoblast differentiation and promoted chondrocyte differentiation. Ectopic cartilage formation has been also reported in the perichondrium of *Osterix*-null mice (Nakashima et al., 2002) and in the perichondrium of mice in which beta-catenin was disrupted in mesenchymal cells (Day et al., 2005; Hill et al., 2005). While normal *Osterix* expression was observed in *ERK1 -/-; ERK2 flox/flox; Prx1-Cre* mice, beta-catenin protein levels were decreased in the perichondrium of *ERK1 -/-; ERK2 flox/flox; Prx1-Cre* mice. These observations suggest a role for decreased beta-catenin in the ectopic cartilage formation in the perichondrium.
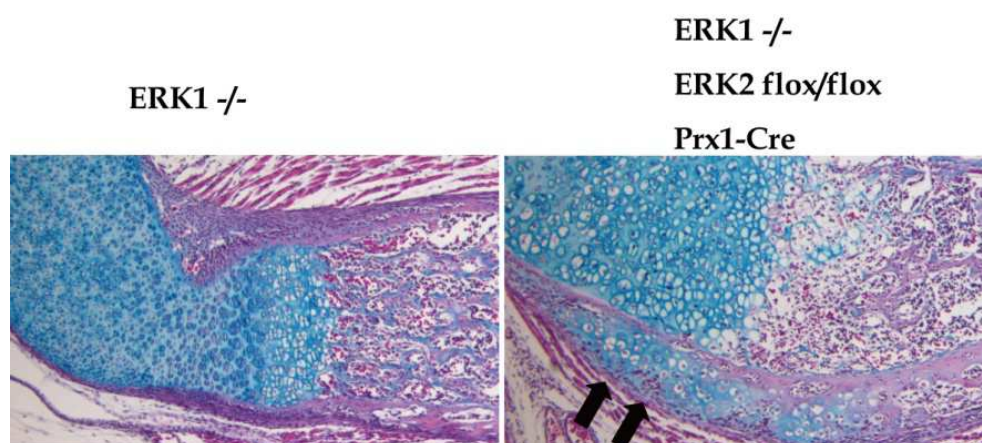

Fig. 3. Hematoxylin, eosin and alcian blue staining of femur of newborn mice. Arrows indicate ectopic cartilage formation in the perichondrium of *ERK1-/-;ERK2*flox/flox;*Prx1-Cre* mice.

6. Constitutive activation of MEK1 in undifferentiated mesenchymal cells leads to increased bone formation and inhibition of cartilage formation

As a complementary experiment, we generated *Prx1-MEK1* transgenic mice that express a constitutively active mutant of MEK1 in undifferentiated limb and cranial mesenchyme (Matsushita et al., 2009a). *Prx1-MEK1* mice showed a marked increase in cortical bone formation, fusion of long bones as well as carpal and tarsal bones, and an accelerated closure of cranial sutures, mimicking the phenotype of human craniosynostosis syndromes caused by activating mutations in FGFR2. The increase in bone formation was associated with increased expression of osteoblast markers, such as *Runx2, Osterix, Bsp*, and *Osteocalcin*. In contrast, cartilage formation was inhibited in *Prx1-MEK1* transgenic mice. There was a clear delay in the formation of cartilage anlagen as well as a decrease in anlagen size. In

Effects on the ERK MAPK pathway	Mouse models	Phenotypes
	Mesenchymal cells	
Loss of function	ERK1 $^{-/-}$; ERK2 $^{flox/flox}$; Prx1-Cre (Matsushita 2009a)	Limb deformity Delayed closure of the cranial suture Calvaria defect Inhibition of bone formation Ectopic cartilage formation in perichondrium Increase in terminally differentiated hypertrophic chondrocytes Absence of Osteocalcin expression Decreased Dmp1 expression Absence of osteocyte-lacunar-canalicur system
Gain of function	CA MEK1/Prx1 (Matsushita 2009a)	Increase in cortical bone formation Fusion of long bones, carpal and tarsal bones Accelerated closure of cranial suture Delayed and decreased formation of cartilage anlagen Increased Runx2, Osterix, Bsp, Osteocalcin expression
	Chondrocytes	
Loss of function	ERK1 $^{-/-}$; ERK2 $^{flox/flox}$; Col2a1-Cre (Matsushita 2009a)	Die immediately after birth Deformed rib cage Kyphotic deformity of the spine Disorganization of epiphyseal cartilage Widening of zone of hypertrophic chondrocytes
	ERK1 $^{-/-}$; ERK2 $^{flox/flox}$; Col2a1-CreER (Sebastian 2011)	Delayed synchondrosis closure of the vertebrae Increased vertebral foramen cross-sectional area
Gain of function	CA MEK1/Col2a1 (Murakami 2004)	Dwarfism Premature synchondrosis closure Narrower zone of hypertrophic chondrocytes Smaller hypertrophic chondrocytes Reduced rate of chondrocyte hypertrophy
	Osteoblasts	
Loss of function	DN MEK1/Osteocalcin (Ge 2007)	Delayed calvarial mineralization Delayed formation of primary ossification centers in the long bones
Gain of function	CA MEK1/Osteocalcin (Ge 2007)	Increased calvarial mineralization Accelerated trabecular bone formation

Table 1. Genetically engineered mouse models with increased or decreased ERK MAPK signaling in skeletal cells. CA MEK1/Prx1, Transgenic mice that express a constitutively active mutant of MEK1 under the control of a *Prx1* promoter. CA MEK1/Col2a1, Transgenic mice that express a constitutively active mutant of MEK1 under the control of a *Col2a1* promoter. DN MEK1/Osteocalcin, Transgenic mice that express a dominant-negative mutant of MEK1 under the control of an *Osteocalcin* promoter. CA MEK1/Osteocalcin, Transgenic mice that express a constitutively active mutant of MEK1 under the control of an *Osteocalcin* promoter.

addition, expression of *Col2a1* was reduced in the cartilage primordia. These observations are consistent with the phenotypes of *ERK1 -/-; ERK2 flox/flox; Prx1-Cre* mice, in which the lack of *ERK1* and *ERK2* disrupts bone formation and induces ectopic cartilage formation. These observations are also consistent with the in vitro studies showing inhibitory effects of the ERK MAPK pathway on chondrogenesis. The ERK MAPK inhibitors U0126 and PD98059 increased the expression of *Col2a1* and *aggrecan* in embryonic limb mesenchyme, and transfection of limb mesenchyme with constitutively active mutant of MEK decreased the activity of a Sox9-responsive *Col2a1* enhancer reporter gene (Bobick & Kulyk, 2004). Collectively, these observations indicate that ERK MAPK signaling plays an important role in the lineage specification of mesenchymal cells.

7. Inactivation of ERK1 and ERK2 in chondrocytes causes severe chondrodysplasia and enhances bone growth

Type II collagen is the most abundant collagen in cartilage. *Col2a1*, the gene encoding the proalpha1(II) collagen chain, is a principal marker of chondrocyte differentiation. We created a loss of function model of ERK1 and ERK2 in chondrocytes by using the *Col2a1-Cre* transgenic mice that express Cre recombinase under the regulatory sequences of *Col2a1* (Matsushita et al., 2009a; Ovchinnikov et al., 2000). *ERK1 -/-; ERK2 flox/flox; Col2a1-Cre* mutant mice died immediately after birth, likely secondary to respiratory insufficiency caused by rib cage deformity (Matsushita et al., 2009a). To circumvent the perinatal lethality, we also used the *Col2a1-CreER* transgene to express a tamoxifen-inducible form of Cre recombinase and examined the role of *ERK1* and *ERK2* in chondrocytes during postnatal growth (Nakamura et al., 2006; Sebastian et al., 2011). For the gain-of-function experiments, we generated *Col2a1-MEK1* transgenic mice that express a constitutively active mutant of MEK1 in chondrocytes under the control of the regulatory sequences of *Col2a1* (Murakami et al., 2004).

7.1 ERK1 and ERK2 are essential for proper organization of epiphyseal cartilage

A strong skeletal phenotype was observed in *ERK1 -/-; ERK2 flox/flox; Col2a1-Cre* embryos. We observed severe kyphotic deformities of the spine. Histological analysis of *ERK1 -/-; ERK2 flox/flox; Col2a1-Cre* embryos at embryonic day 16.5 showed an absence of primary ossification centers in the axial skeleton and a widening of the zone of hypertrophic chondrocytes in the long bones (Fig. 4). In addition, disorganization of the epiphyseal cartilage with lack of columnar growth structures was observed in *ERK1 -/-; ERK2 flox/flox; Col2a1-Cre* embryos at embryonic day 18.5. These findings indicate that ERK1 and ERK2 are essential for the proper organization of the epiphyseal cartilage.
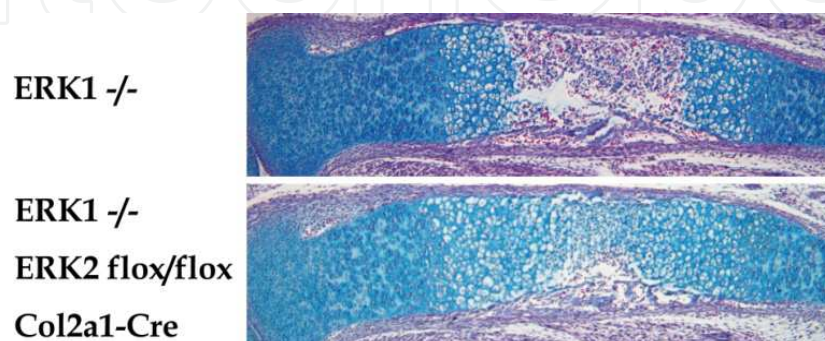

Fig. 4. Hematoxylin, eosin and alcian blue staining of the tibia showed delayed formation of primary ossification center in *ERK1 -/-; ERK2 flox/flox; Col2a1-Cre* embryos at embryonic day 16.5.

7.2 ERK1 and ERK2 inhibit hypertrophic chondrocyte differentiation

Both *ERK1 -/-; ERK2 flox/flox; Prx1-Cre* and *ERK1 -/-; ERK2 flox/flox; Col2a1-Cre* mice showed a remarkable expansion of the zone of hypertrophic chondrocytes. In both animal models, chondrocytes that were closer to the articular surface expressed hypertrophic chondrocyte marker *Col10a1*, suggesting premature chondrocyte hypertrophy. These observations are consistent with the growth plate phenotype of *Col2a1-MEK1* transgenic mice that express a constitutively active MEK1 mutant in chondrocytes (Murakami et al., 2004). The growth plate of *Col2a1-MEK1* transgenic mice was characterized by smaller than normal hypertrophic chondrocytes and narrower zone of hypertrophic chondrocytes. BrdU labeling of proliferating chondrocytes and subsequent identification of BrdU-labeled hypertrophic chondrocytes indicated reduced rate of chondrocyte hypertrophy in *Col2a1-MEK1* mice. Collectively, these observations indicate that ERK MAPK signaling inhibits hypertrophic chondrocyte differentiation.

The pronounced expansion of the zone of hypertrophic chondrocytes in *ERK1 -/-; ERK2 flox/flox; Prx1-Cre* mice was also characterized by an increase in terminally differentiated hypertrophic chondrocytes expressing *Vegf*, *Mmp13*, and *Osteopontin,* suggesting impaired removal of terminally differentiated hypertrophic chondrocytes. We also observed a decrease in TRAP-positive osteoclasts in association with reduced expression of *receptor activator of nuclear factor-kappa B ligand (RANKL).* Therefore, decreased osteoclastogenesis may also account for the expansion of the zone of hypertrophic chondrocytes.

7.3 ERK1 and ERK2 inhibit growth of cartilaginous skeletal element

A number of genetic studies have indicated the growth inhibitory role of FGFR3 signaling. Mice with activating mutations in Fgfr3 show a dwarf phenotype similar to the human syndromes of achondroplasia and thanatophoric dysplasia (Chen et al., 1999; Iwata et al., 2000, 2001; Li et al., 1999; Naski et al., 1998; Wang et al., 1999). In contrast, *Fgfr3*-null mice show a skeletal overgrowth (Colvin et al., 1996; Deng et al., 1996). Our observations in genetically engineered mouse models have provided evidence indicating that the ERK MAPK pathway is a critical downstream effector of Fgfr3 signaling. We have shown that *Col2a1-MEK1* transgenic mice that express a constitutively active MEK1 mutant in chondrocytes show an achondroplasia-like dwarf phenotype (Murakami et al., 2004). We have also shown that *ERK1* and *ERK2* inactivation in chondrocytes promotes bone growth. We found increased length of the proximal long bones, specifically the humerus and femur, of *ERK1 -/-; ERK2 flox/flox; Col2a1-Cre* embryos (Sebastian et al., 2011). These embryos also showed an increase in the width of epiphyses in the humerus and femur. Histological analysis of the vertebrae also showed an overgrowth of cartilage in the vertebral body. These observations indicate that ERK1 and ERK2 negatively regulate the growth of cartilaginous skeletal elements.

8. Postnatal ERK1 and ERK2 inactivation delays synchondrosis closure and enlarges the spinal canal

Our studies have indicated that FGFR3 and the MAPK pathway are important regulators of synchondrosis closure. In bones such as the vertebrae, sternum, pelvis, and bones in the cranial base, a synchondrosis—a growth plate-like cartilaginous structure—connects the

ossification centers and contributes to the bone growth. During postnatal skeletal development, the width of synchondroses reduces with age. Ossification centers eventually unite when synchondroses close. Histologically, a synchondrosis consists of two opposed growth plates with a common zone of resting chondrocytes (Fig. 5A). We have found premature synchondrosis closure in the vertebrae and cranial base of human samples of achondroplasia and thanatophoric dysplasia (Matsushita et al., 2009b). In addition, we have also observed premature synchondrosis closure in a mouse model of achondroplasia and *Col2a1-MEK1* transgenic mice that express a constitutively active MEK1 mutant in chondrocytes (Fig. 5B). Because growth at the synchondrosis determines the final dimension and shape of the endochondral skeletons, premature synchondrosis closure should play a critical role in the development of spinal canal stenosis that is frequently seen in patients with achondroplasia.

Since increased Fgfr3 and MEK1 signaling accelerates synchondrosis closure, we hypothesized that *ERK1* and *ERK2* inactivation delays synchondrosis closure and enlarges the spinal canal. To test this hypothesis, we inactivated *ERK2* in chondrocytes of *ERK1*-null
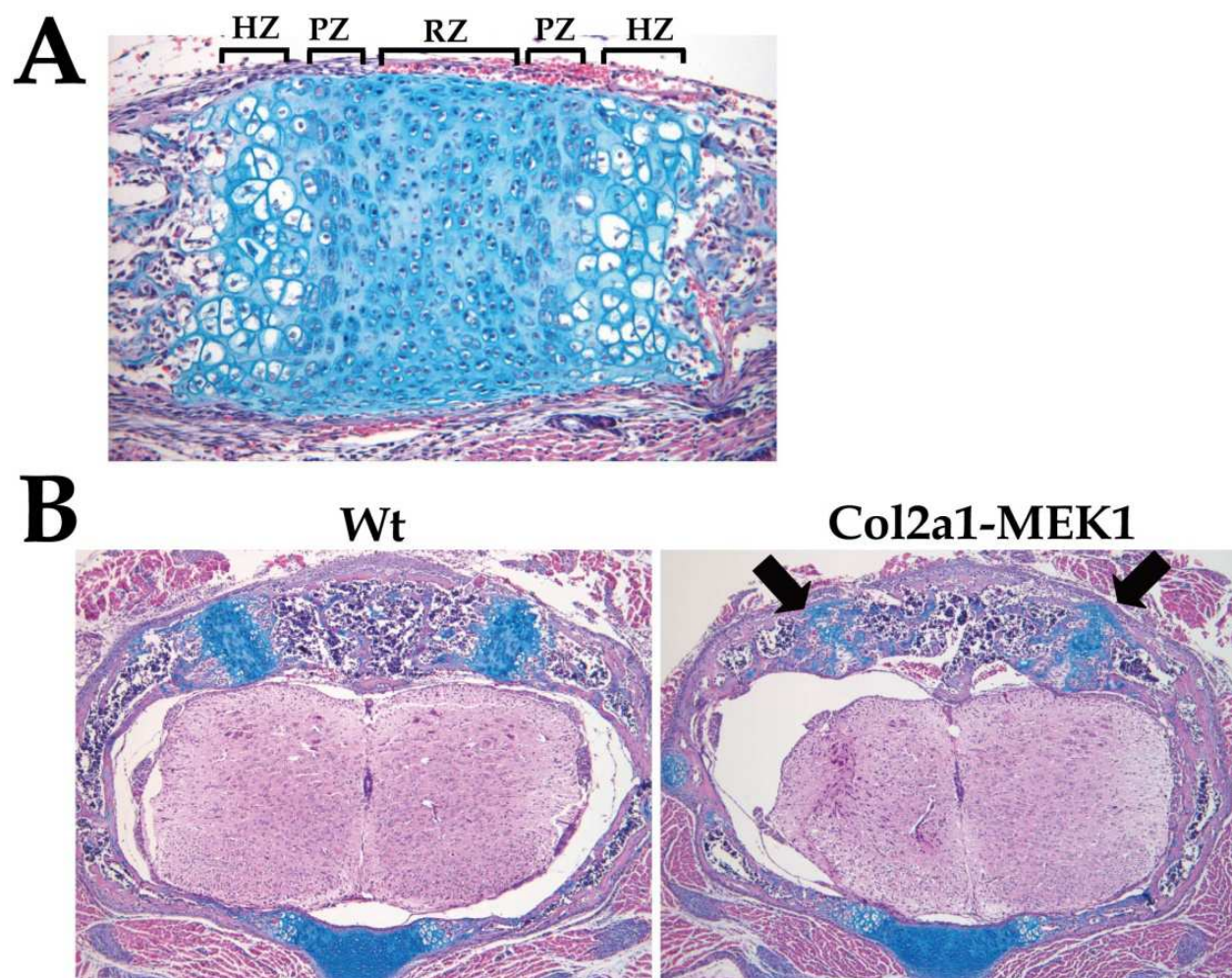

Fig. 5. (A) Spheno-occipital synchondrosis of a 4-day-old wild type mouse. HZ; hypertrophic zone. PZ; proliferation zone. RZ; resting zone. (B) Thoracic spine of wild type and *Col2a1-MEK1* transgenic mice at postnatal day 4. Arrows indicate prematurely closing synchondroses. Wt; wild type.

mice using the *Col2a1-CreER* transgene (Sebastian et al., 2011). Tamoxifen injection into *ERK1 -/-; ERK2 flox/flox; Col2a1-CreER* mice resulted in 60% inhibition of *ERK2* expression in the epiphyseal cartilage. Although these mice did not show an increased growth of the long bones presumably due to incomplete *ERK2* inactivation, we observed a significant delay in synchondrosis closure of the vertebrae and an increase in the cross-sectional area of vertebral foramen. The delayed synchondrosis closure was associated with a decreased expression of the endothelial marker CD31 surrounding the synchondroses, suggesting that *ERK1* and *ERK2* inactivation in chondrocytes causes reduced vascular invasion. These observations indicate the potential of ERK1 and ERK2 as therapeutic targets for spinal canal stenosis in achondroplasia.

9. Conclusion

By creating and analyzing gain-of-function and loss-of-function mouse models, we have identified multiple roles of the ERK MAPK pathway at successive steps of skeletal development. In undifferentiated mesenchymal cells, *ERK1* and *ERK2* inactivation causes a block in osteoblast differentiation and induces ectopic cartilage formation. In contrast, increased MEK1 signaling promotes bone formation and inhibits cartilage formation. These observations indicate that ERK MAPK signaling plays a critical role in the lineage specification of mesenchymal cells (Fig. 6). ERK MAPK signaling also inhibits hypertrophic chondrocyte differentiation and bone growth. Furthermore, ERK MAPK signaling regulates the timing of growth plate and synchondrosis closure, the very last step of endochondral ossification. A better understanding of the roles of the ERK MAPK pathway in skeletal tissues will lead to new insights in skeletal development and the treatment of various skeletal disorders.
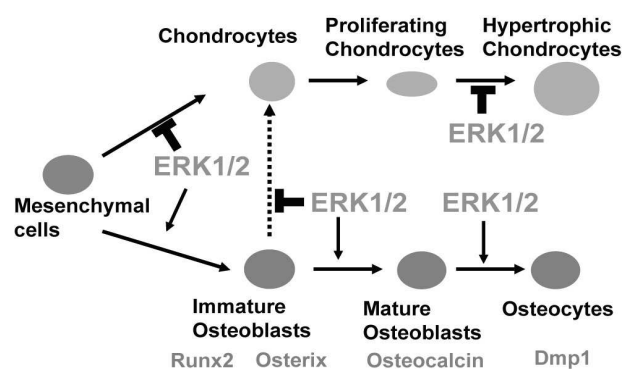

Fig. 6. Proposed model of the role of ERK1 and ERK2 in the regulation of osteoblast and chondrocyte differentiation.

10. Acknowledgments

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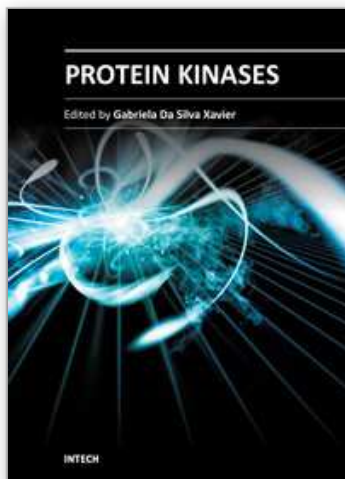
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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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