FULL LENGTH ARTICLE

Highly expressed BMP9/GDF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism

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Abstract   Bone morphogenetic protein 9 (BMP9) (or GDF2) was originally identified from fetal mouse liver cDNA libraries. Emerging evidence indicates BMP9 exerts diverse and pleiotropic functions during postnatal development and in maintaining tissue homeostasis. However, the expression landscape of BMP9 signaling during development and/or in adult tissues remains to be analyzed. Here, we conducted a comprehensive analysis of the expression landscape of BMP9 and its signaling mediators in postnatal mice. By analyzing mouse ENCODE transcriptome datasets we found Bmp9 was highly expressed in the liver and detectable in embryonic brain, adult lungs and adult placenta. We next conducted a comprehensive qPCR analysis of RNAs isolated from major mouse tissues/organs at various ages. We found that Bmp9 was highly expressed in the liver and lung tissues of young adult mice, but decreased in older mice. Interestingly, Bmp9 was only expressed at low to modest levels in developing bones. BMP9-associated TGFβ/BMPR type I receptor Alk1 was highly expressed in the adult lungs. Furthermore, the feedback inhibitor Smads Smad6 and Smad7 were widely expressed in mouse postnatal tissues. However, the BMP signaling antagonist noggin was highly expressed in fat and heart in the older age groups, as well as in kidney, liver and lungs in a biphasic fashion. Thus, our findings indicate that the circulating BMP9 produced in liver and lungs may account for its pleiotropic effects on postnatal tissues/organs although possible roles of BMP9 signaling in liver and lungs remain to be fully understood.

Introduction

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and play important roles in embryogenesis, organogenesis, cell proliferation, stem cell differentiation, and adult tissue homeostasis.[1–10] Disruptions in BMP signaling cause skeletal and extraskeletal anomalies.[1,4,11] At least 14 types of BMPs in humans and rodents have been identified.[1,5,9,12,13] BMPs were initially discovered when demineralized bone was found to induce *de novo* bone formation.[14,15]

Different BMPs exert distinct but overlapping biological functions. BMP9 (also known as growth differentiation factor 2 or GDF-2) represents a less characterized member of the BMP family.[4,9,13] Through a comprehensive analysis of human BMPs in mesenchymal stem cells (MSCs), we demonstrated that BMP9 is one of the most potent BMPs among the 14 types of BMPs in inducing osteogenic/odontogenic differentiation of MSCs.[9,10,16–19] We also found that BMP9 is resistant to the naturally occurring antagonist noggin,[20] and demonstrated that TGFβ/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in MSCs.[21] As BMP9 is one of the least studied BMP, we performed mechanism-based studies and

identified several early downstream targets,[9,10,22–28] and demonstrated that BMP9 signaling has extensive cross-talks with other signaling pathways, especially Wnt and Notch signaling.[9,28–37]

It has also been reported that BMP9 plays important roles in inducing and maintaining basal forebrain cholinergic neurons, inhibiting hepatic glucose production, inducing the expression of key enzymes of lipid metabolism, stimulating hepcidin 1 expression, regulating angiogenesis, and modulating tumorigenesis.[10,38] BMP9 has further been implicated in the pathogenesis of pulmonary arterial hypertension.[38,39]

Originally identified from fetal mouse liver cDNA libraries, BMP9 is highly expressed in the developing mouse liver.[4,9,40] Increasing evidence indicates that BMP9 exerts diverse and pleiotropic biological functions during postnatal development and in maintaining adult tissue homeostasis. Thus, it is conceivable that a postnatal expression landscape of BMP9 and its important signaling mediators would provide important insights into the potential functions of BMP9 signaling in major organs and/or tissues. However, the expression landscape of BMP9 and its signaling mediators during development and/or in adult tissues has yet to be fully investigated.

In this study, we sought to conduct a comprehensive analysis of the expression landscape of BMP9 and its signaling mediators in postnatal mice. We first analyzed the mouse ENCODE transcriptome data sets and found that Bmp9 was highly expressed in liver although Bmp9 expression was detectable in embryonic brain, adult lungs and adult placenta. However, the ENCODE datasets are limited in scope and depth. Thus, we conducted a comprehensive qPCR analysis of the RNAs isolated from major mouse tissues/organs at various ages. Our results demonstrated that, although at relatively lower levels at birth, Bmp9 was highly expressed in the liver and lung tissues of young adult mice, but decreased in older mice. Bmp9 expression was also detectable in 2-week spleen, brain and fat tissues and muscle samples. Interestingly, Bmp9 was only expressed at modest to low levels in developing bones. Alk1 was highly expressed in the adult lungs, while Alk2 was expressed at relatively higher levels in fat and kidney tissues. Furthermore, the feedback inhibitors Smad6 and Smad7 of BMP signaling were widely expressed in mouse postnatal tissues, including developing bones. However, the BMP signaling antagonist noggin was expressed highly in fat and heart tissues in the older age groups, as well as in kidney, liver and lung tissues in a biphasic fashion. Collectively, our findings indicate that the circulating BMP9 produced in liver and lungs may account for its pleiotropic effects on multiple postnatal tissues/organs although the functional roles of BMP9 signaling in liver and lungs remain to be fully understood.

Materials and methods

Mouse ENCODE transcriptome data analysis

The RNA profiling data sets were generated by the Mouse ENCODE project PRJNA66167 as described.[41] The data sets included 30 mouse samples derived from embryonic and adult tissues, and are publically available through NCBI website (https://www.ncbi.nlm.nih.gov/gene/). The acquired reads per kilobase per million reads (RPMKs) for Bmp9, Alk1, Alk2, Smad6 and Smad7 in the 30 mouse samples were graphed by using the Microsoft Excel software.

Animal tissue samples

The use of mouse tissues was approved by the Institutional Animal Care and Use Committee and all experimental procedures involved in harvesting mouse tissues were performed according to the approved guidelines. Briefly, C57BL/6J mice at newborn (NB), 2-week, 4-week, 3-month, 8-month and 18-month old (male, n = 5) were obtained from The University of Chicago Transgenic Core Facility.

Total RNA isolation from mouse tissues and reverse transcription

For total RNA isolation, mouse brain, fat (inguinal region), heart, kidney, liver, lung, muscle, spleen, femur and parietal bone (PB) at various ages were harvested immediately after sacrificing the animals, placed in RNase-free mortars containing NucleoZOL Reagent (Takara Bio USA, Mountain View, CA) and liquid nitrogen, and crashed with RNase-free pestles in a RNase-free biosafety cabinet. Total RNA was subsequently isolated according to the manufacturer's introduction, and subjected to reverse transcription with hexamer and M-MuLV (New England Biolabs, Ipswich, MA). The cDNA products were diluted as templates for qPCR.

Touchdown quantitative real-time PCR (TqPCR)

The qPCR primers were designed by using Primer3 Plus program.[42] SYBR Green-based quantitative real-time PCR analysis was performed by following our previously optimized TqPCR protocol.[43] The qPCR reactions were done in triplicate. All expression values were normalized to the reference gene Gapdh expression by using the $2^{-\Delta\Delta Ct}$ method.[44–47] The qPCR primer sequences are listed in Table 1.

Results and discussion

Mouse ENCODE transcriptome data indicate Bmp9 is highly expressed in liver while Bmp9-associated BMP type I receptor Alk1 is highly expressed in lungs

As one of the least studied BMPs, BMP9 was originally identified from mouse embryonic liver tissue.[40] However, the expression patterns of BMP9 and its signaling mediators during development and/or in adult tissues have not been investigated. The recently completed ENCODE transcriptome projects have offered a glimpse of the expression patterns for a given gene.

Table 1 List of qPCR Primers.

Gene	Accession No.	Forward	Reverse
Bmp9/Gdf2	NM_019506.4	TGAGTCCCATCTCCATCCTC	ACCCACCAGACACAAGAAGG
Alk1	NM_001277255.1	ACCTGGGACTGGCTGTGA	GCAGTCTGTGCGGATGTG
Alk2	NM_001110204.1	GTGGCTCCGGTCTTCCTT	AGCGACATTTTCGCCTTG
Smad6	NM_008542.3	ATCACCTCCTGCCCCTGT	CTGGGGTGGTGTCTCTGG
Smad7	NM_001042660.1	AAGATCGGCTGTGGCATC	CCAACAGCGTCCTGGAGT
Nogging	NM_008711.2	GCGGCCAGCACTATCTACA	GGGGCGAAGTAGCCATAAA
Gapdh	NM_008084	GAAGGTCGGTGTGAACGGAT	ACTGTGCCGTTGAATTTGCC

Here, we took advantage of the mouse ENCODE database and examined the expression patterns of Bmp9 and its signaling mediators in 30 mouse samples. Consistent with earlier reports,[40] Bmp9 expression level was apparently high in liver E14, E14.5, E18 and adult liver samples, while Bmp9 expression was also detected in CNS E11.5, adult lung, and adult placenta (Fig. 1A). Surprisingly, Bmp9 associated BMP type I receptor Alk1 was highly expressed in adult lung tissue although several tissues (such as adult bladder, colon, genital fat pad, heart, kidney, mammary gland, ovary, and subcutaneous fat pad) expressed detectable levels of Bmp9 (Fig. 1B). Conversely, another Bmp9-associated type I receptor Alk2 exhibited a broad range, even though many of those tissues exhibited medium to lower levels of Bmp9 expression (Fig. 1C).

We also analyzed the ENCODE data for BMP signaling feedback inhibitor Smads (or I-Smads), Smad6 and Smad7. Surprisingly, adult lung tissue has the highest expression for both I-Smads, followed by various GI tissues, although Smad7 is more widely expressed than Smad6 (Fig. 1D & E). Taken together, the mouse ENCODE transcriptome data indicate that Bmp9 expression is relatively restricted to liver, and to a lesser extent, lungs, while the Bmp9 signaling mediators, especially Alk1, Smad6 and Smad7, exhibit high expression levels in adult lungs, suggesting that BMP9 may play an important role in regulating biological functions and tissue homeostasis of the lungs.

Bmp9 is highly expressed in postnatal liver and lung tissues

Even though the ENCODE database has offered a quick overview of the expression of Bmp9 and its signaling mediators, those data are limited in scope and depth. On the other hand, the currently available antibodies against BMP9
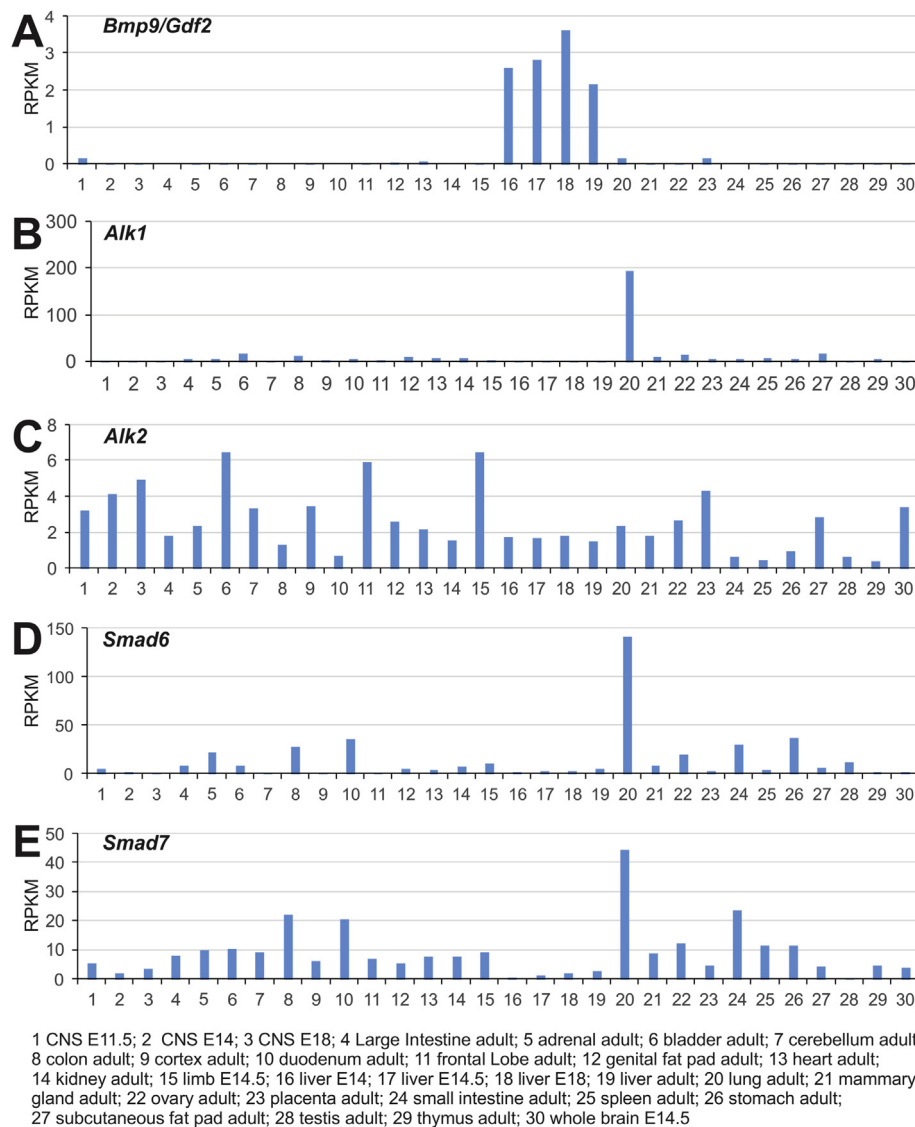

1 CNS E11.5; 2 CNS E14; 3 CNS E18; 4 Large Intestine adult; 5 adrenal adult; 6 bladder adult; 7 cerebellum adult; 8 colon adult; 9 cortex adult; 10 duodenum adult; 11 frontal Lobe adult; 12 genital fat pad adult; 13 heart adult; 14 kidney adult; 15 limb E14.5; 16 liver E14; 17 liver E14.5; 18 liver E18; 19 liver adult; 20 lung adult; 21 mammary gland adult; 22 ovary adult; 23 placenta adult; 24 small intestine adult; 25 spleen adult; 26 stomach adult; 27 subcutaneous fat pad adult; 28 testis adult; 29 thymus adult; 30 whole brain E14.5

Figure 1   Across-tissue expression of *Bmp9* and its signaling mediators revealed by mouse ENCODE transcriptome analysis. The RNA profiling data sets (n = 30 samples) were generated by the Mouse ENCODE project, PRJNA66167, as described in.[41] The tabulated reads per kilobase per million reads (RPMKs) for *Bmp9* (**A**), *Alk1* (**B**), *Alk2* (**C**), *Smad6* (**D**) and *Smad7* (**E**) in the 30 mouse samples were graphed. The individual samples were delineated at the bottom of the graphs.

lack either specificity and/or reactivity. Furthermore, it is relatively difficult to quantify immunostaining results. Thus, we decided to carry out qPCR analysis of the post-natal expression of Bmp9 and related signaling mediators. We collected total RNA from the major mouse tissues/organs, such as whole brain, fat (inguinal region), heart, kidney, liver, lungs, muscle, spleen, femur, and parietal bone (PB) at various ages, including newborn (NB), 2-week, 4-week, 3-month, 8-month and 18-month old mice.

Using our previously optimized touchdown qPCR (or TqPCR) technique,[43] we analyzed the expression of Bmp9 in these tissues. At age groups, Bmp9 was shown to express at much higher levels in liver and lung tissues, although its expression showed an age-dependent trend in decrease (Fig. 2A). Interestingly, a significant expression peak of Bmp9 was found in 2-week spleen tissue (Fig. 2A). A detailed analysis indicates that Bmp9 expression was readily detectable in 2-week brain, 2-week and 8-month fat tissue, and in muscle at most of the analyzed time points (Fig. 2B, panels a, b, and e). Interestingly, even though highly expressed at later time points (e.g., 2-week, 4-week and 3-month), Bmp9 was expressed at relatively low levels in both liver and lung tissues at birth (Fig. 2B, panels c & d). In bone tissue such as femur, Bmp9 expression increased over the time from newborn to 3-month (Fig. 2B, panel f),

indicating BMP9 may play an important role in bone and skeletal development.

Bmp9-associated TGFβ/BMPR type I receptor Alk1 is highly expressed in the lungs, while Alk2 expressed relatively higher in fat and kidney tissues

We previously demonstrated that BMP9 initiates its signaling through interactions with TGFβ/BMPR type I receptor Alk1 and Alk2[21]. Our results indicated that Alk1 was modestly expressed in various age groups of the brain, heart, kidney and liver tissues (Fig. 3A, panels a, c, d, and e). However, the highest expression levels of Alk1 were found in the lungs, especially in 3-month lungs, although Bmp9 was also highly expressed in 8-month fat tissue (Fig. 3A, panels c & f). On the other hand, Alk2 was expressed modestly, but more broadly in the analyzed tissues, including brain, fat, heart, kidney, liver and lungs, although the highest level of Alk2 expression was found in 8-week fat tissue sample (Fig. 3B, panels a to f).

Lastly, we examined the expression of Alk1 and Alk2 in the developing bone tissues, femurs and parietal bone (PB). We found that the expression of Alk1 and Alk2 peaked at the 4-week time point in both bone samples (Fig. 3C, panels
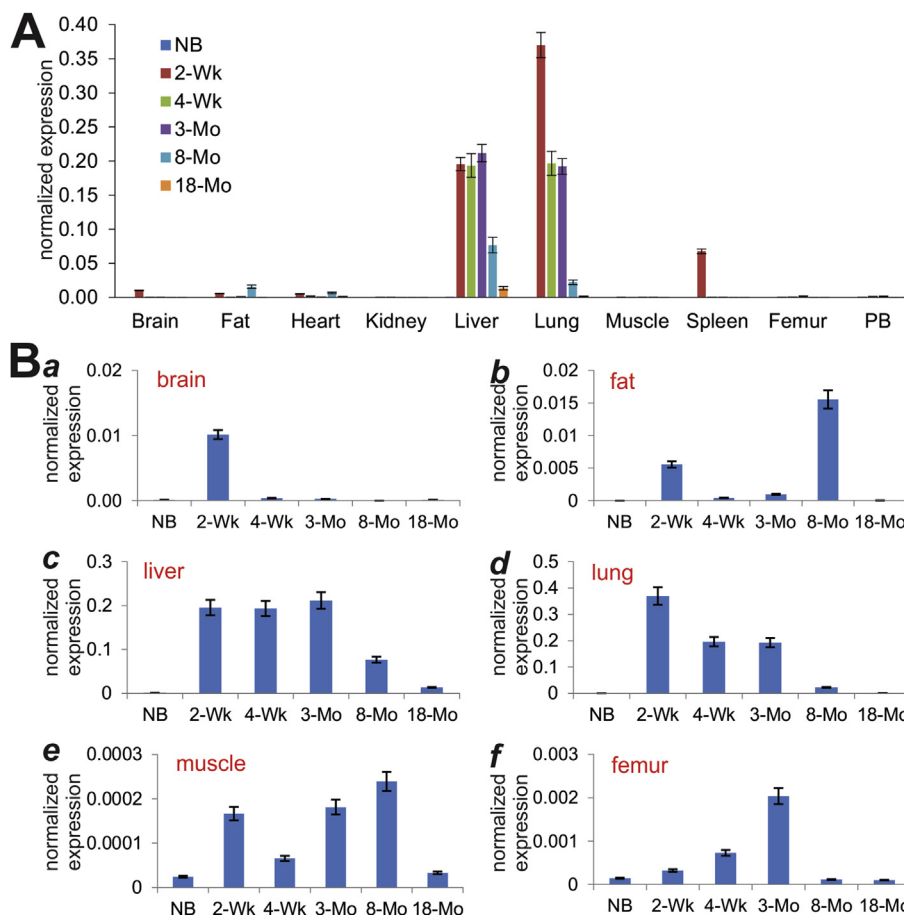

Figure 2 Expression of Bmp9 in postnatal mouse tissues determined by TqPCR analysis. Total RNA was isolated from mouse brain, fat (inguinal region), heart, kidney, liver, lung, muscle, spleen, femur, and parietal bone (PB) at various ages: newborn (NB), 2-week, 4-week, 3-month, 8-month and 18-month old. The RT cDNA products were subjected to TqPCR analysis of Bmp9 expression in these tissues.
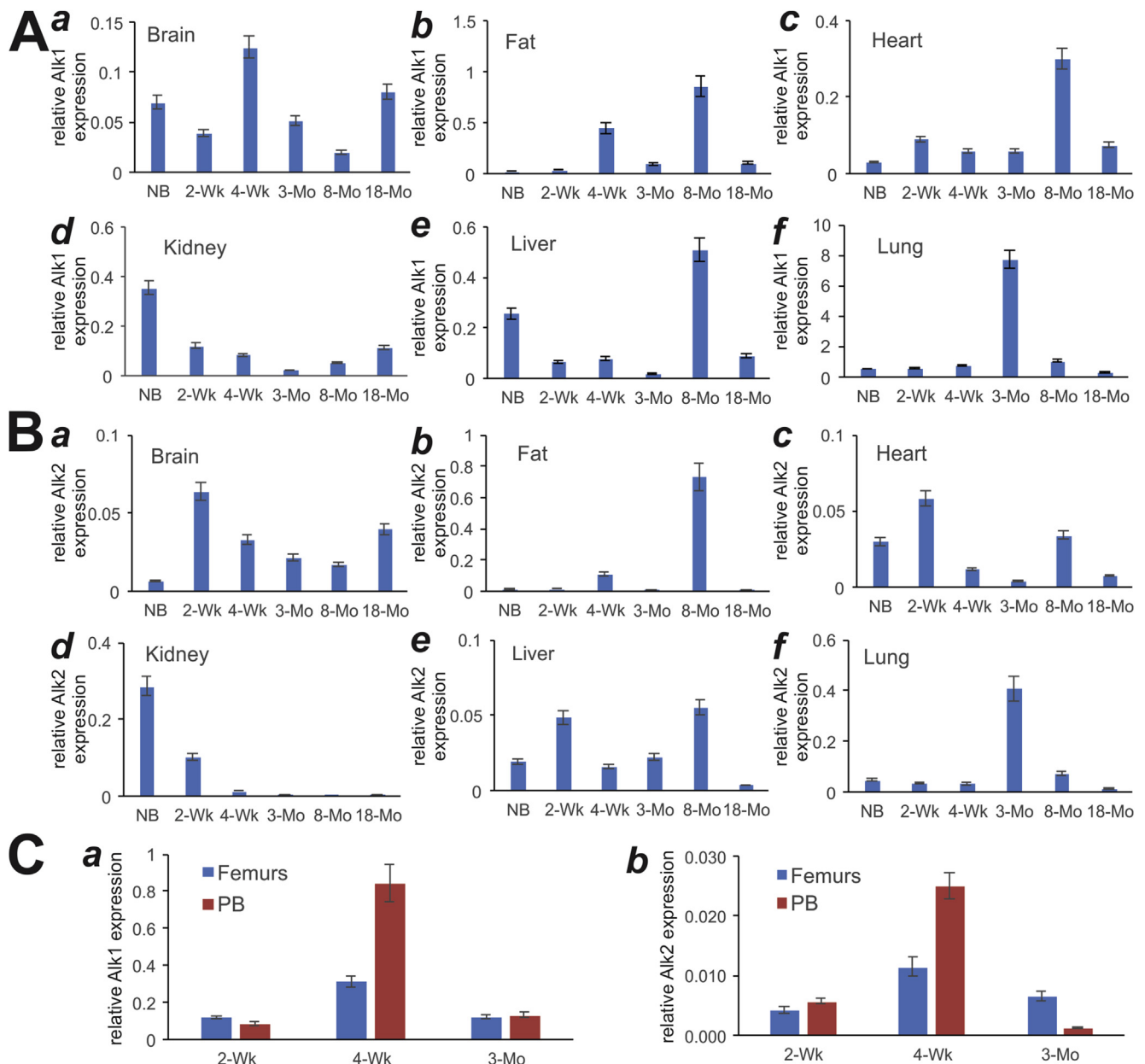
Figure 3 Expression of BMP9-associated receptors *Alk1* and *Alk2* in postnatal mouse tissues determined by TqPCR analysis. Total RNA was isolated from mouse brain, fat (inguinal region), heart, kidney, liver, lung, muscle, spleen, femur, and parietal bone (PB) at various ages: newborn (NB), 2-week, 4-week, 3-month, 8-month and 18-month old. The RT cDNA products were subjected to TqPCR analysis of *Alk1* and *Alk2* expression in these tissues.

a & b). However, *Alk1* was expressed at much higher levels than *Alk2* at the three age groups, suggesting *Alk1* may play a more important role in developing bones.

The negative regulators of BMP9 signaling, Smad6, Smad7 and noggin, are widely expressed in mouse postnatal tissues

As the feedback inhibitors Smad6 and Smad7 (or so-called I-Smads) can serve as indirect indicators of the status of BMP signaling activities including BMP9, we further analyzed the expression patterns of Smad6 and Smad7 in the mouse tissues. In brain tissue, the expression of both Smad6 and

Smad7 was shown highest in the 4-week group (Fig. 4A panel a and Fig. 4B panel a). On the other hand, both Smad6 and Smad7 peaked their expression in the 8-month age group in fat, heart, and liver tissues (Fig. 4A panels b, c, & e, and Fig. 4B panels b, c, & e). Smad6 and Smad7 also exhibited similar expression patterns in kidney samples with the highest expression at the 2-week age group (Fig. 4A panel d, and Fig. 4B panel d). However, the expression of Smad6 and Smad7 in the lungs seemingly increased with ages within the first 8 and 3 months after birth, respectively (Fig. 4A, panel f; and Fig. 4B panel f).

We further analyzed the expression of I-Smads in developing bone tissues and found that both *Smad6* and *Smad7* peaked their expression in femurs and parietal bone
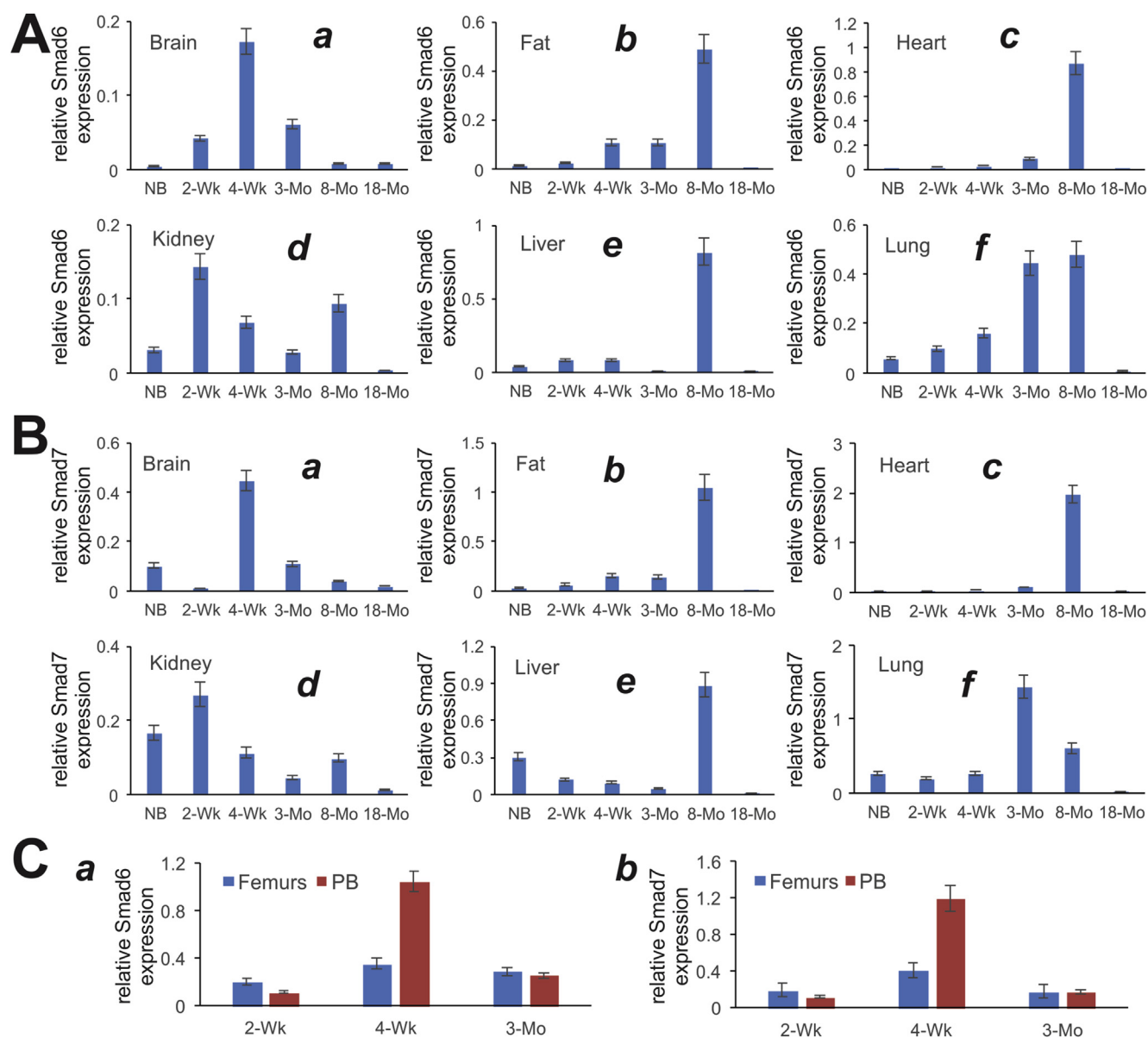
Figure 4 Expression of BMP9 signaling feedback inhibitors, *Smad6* and *Smad7*, in postnatal mouse tissues determined by TqPCR analysis. Total RNA was isolated from mouse brain, fat (inguinal region), heart, kidney, liver, lung, muscle, spleen, femur, and parietal bone (PB) at various ages: newborn (NB), 2-week, 4-week, 3-month, 8-month and 18-month old. The RT cDNA products were subjected to TqPCR analysis of *Smad6* and *Smad7* expression in these tissues.

at the 4-week age group (Fig. 4C panels a & b), suggesting that BMP signaling, which is negatively regulated by *Smad6* and *Smad7*, may play an important role in developing bones.

Lastly, we analyzed the expression of the BMP signaling antagonist noggin. We found that noggin was expressed at low levels in the brain tissues (Fig. 5A panel a). In fat and heart tissues, noggin was shown to express at older age groups (e.g., 4-week to 8-month) (Fig. 5A, panels b & c). On the other hand, in kidney, liver and lung tissues noggin showed biphasic expression, expressed at high levels at birth and then dropped sharply with gradual increases with age (Fig. 5A, panels d, e, & f). However, in the developing bone tissues noggin expression increased with ages, from 2-week, 4-week to 3-month in femur and parietal bone

tissues (Fig. 5B, panel a & b). Taken together, these results indicate that negative regulators of BMP9 signaling, Smad6, Smad7 and noggin, are widely expressed in mouse postnatal tissues.

BMP9 exhibits pleiotropic biological functions and yet is primarily expressed in postnatal liver and lungs

Increasing evidence indicates that BMP9 plays diverse arrays of biological functions in development and adult tissue homeostasis. However, little is known about the *in vivo* expression landscape of BMP9 and its signaling mediators. We analyzed the mouse ENCODE transcriptome dataset,
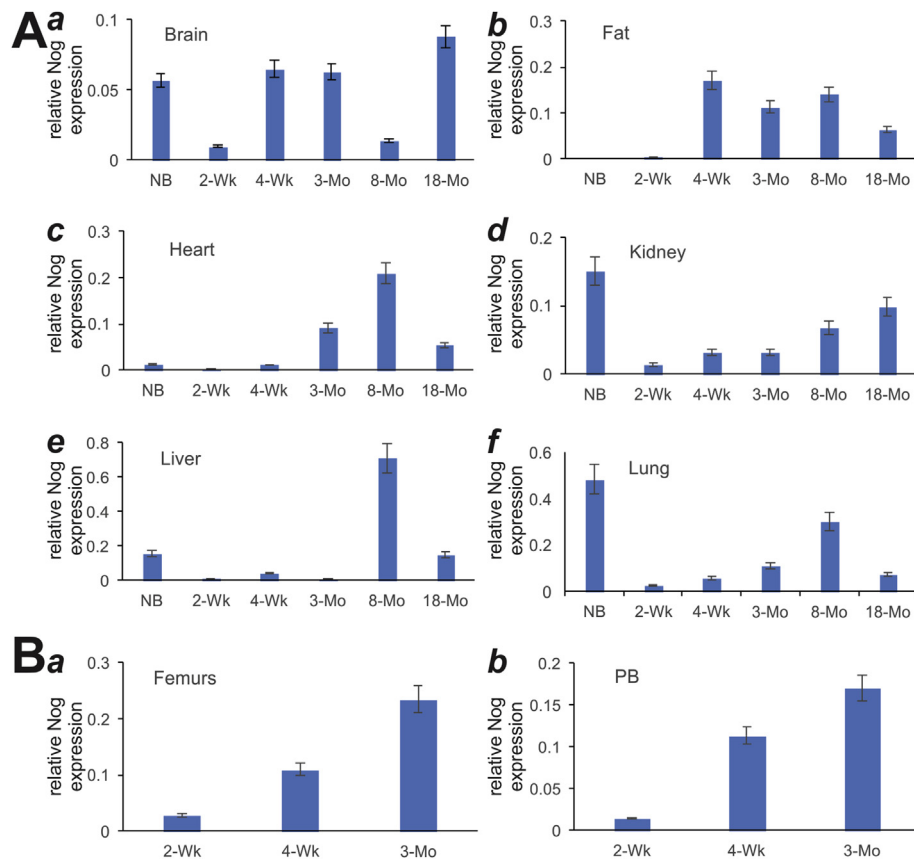
Figure 5 Expression of BMP9 signaling inhibitor, *Noggin*, in postnatal mouse tissues determined by TqPCR analysis. Total RNA was isolated from mouse brain, fat (inguinal region), heart, kidney, liver, lung, muscle, spleen, femur, and parietal bone (PB) at various ages: newborn (NB), 2-week, 4-week, 3-month, 8-month and 18-month old. The RT cDNA products were subjected to TqPCR analysis of *Noggin* expression in these tissues.

which contains 30 mouse samples of embryonic and adult tissues, and found that Bmp9 was highly expressed in E14, E14.5, E18 liver and adult liver, although Bmp9 expression was detectable in E11.5 brain, adult lungs and adult placenta. The ENCODE dataset further revealed that BMP9 receptor Alk1 was highly expressed in adult lung tissue while Alk2 was more broadly expressed, and that adult lung tissue had the highest expression for both I-Smads, followed by various GI tissues, although Smad7 is more widely expressed than Smad6. Thus, the mouse ENCODE transcriptome data suggest that Bmp9 expression may be relatively restricted to liver, and to a lesser extent, lungs, while the Bmp9 signaling mediators, especially Alk1, Smad6 and Smad7, exhibit high expression levels in adult lungs.

To overcome the ENCODE's limitations in scope and depth, we conducted a transcriptomic analysis of BMP9 signaling in major mouse organs including brain, fat, heart, kidney, liver, lungs, muscle, spleen, femur, and parietal bone at different ages ranging from newborn, 2-week, 4-week, 3-month, 8-month to 18-month old mice. Our results demonstrated that, although at relatively low levels at birth, Bmp9 was highly expressed in the liver and lung tissues of young adult mice, but decreased in older mice. Bmp9 expression was also detectable in 2-week spleen, brain and fat tissues and muscle samples. Interestingly, Bmp9 was only expressed at low to modest levels in developing bones.

Furthermore, BMP9-specific BMP-R type I receptor Alk1 was highly expressed in the adult lungs, while Alk2 was expressed at relatively higher levels in fat and kidney tissues. The I-Smads Smad6 and Smad7 were widely expressed in mouse postnatal tissues, including developing bones. The naturally occurring antagonist of BMP signaling, noggin, was expressed highly in fat and heart tissues at older age groups, as well as in kidney, liver and lung tissues in a biphasic fashion. It seems that the expression patterns of noggin were independent of that of BMP9, Alk1 and/or I-Smads in adult mice, which is consistent with our earlier findings about the refractory nature of BMP9 against noggin.[20]

Emerging evidence indicates that BMP9 exerts diverse and pleiotropic biological functions during postnatal development and in maintaining adult tissue homeostasis. The postnatal expression landscape of BMP9 and its important signaling mediators should provide important insights into the potential functions of BMP9 signaling in major organs and/or tissues. We have recently conducted a comprehensive transcriptomic profiling of the MSCs stimulated by the 14 types of BMPs at the early stage, which allows us to identify the immediate early transcriptomic changes in MSCs.[48] The present study indicates that the circulating BMP9 produced in liver and lungs may account for its pleiotropic effects on multiple postnatal tissues/organs. Furthermore, the expression profiling analysis

strongly suggests that BMP9 may play important roles in regulating hepatic metabolism and tissue homeostasis and pulmonary endothelial cells of the lungs,[10,38,39] which warrants thorough future investigations.

Conflict of interest

The authors declare no competing interest.

Acknowledgments

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