Published in final edited form as:

Genesis. 2017 July ; 55(7): . doi:10.1002/dvg.23035.

Characterization of New Bone Morphogenetic Protein (Bmp)-2 Regulatory Alleles

Tapan A. Shah^{1,*}, Youhua Zhu^{1,*}, Nadia N. Shaikh¹, Marie A. Harris², Stephen E. Harris², and Melissa B. Rogers¹

¹Department of Microbiology, Biochemistry, and Molecular Genetics, Rutgers NJMS, Newark, NJ

²Department of Periodontics, University of Texas Health Science Centre, San Antonio, TX

Abstract

Bone morphogenetic protein 2 (BMP2, HGNC:1069, GeneID: 650) is a classical morphogen; a molecule that acts at a distance and whose concentration influences cell proliferation, differentiation, and apoptosis. Key events requiring precise *Bmp2* regulation include heart specification and morphogenesis and neural development. In mesenchymal cells, the concentration of BMP2 influences myogenesis, adipogenesis, chondrogenesis, and osteogenesis. Because the amount, timing, and location of BMP2 synthesis influence pattern formation and organogenesis, the mechanisms that regulate *Bmp2* are crucial. A sequence within the 3'UTR of the *Bmp2* mRNA termed the "ultra-conserved sequence" (UCS) has been largely unchanged since fishes and mammals diverged. Cre-lox mediated deletion of the UCS in a reporter transgene revealed that the UCS may repress *Bmp2* in proepicardium, epicardium and epicardium-derived cells (EPDC) and in tissues with known epicardial contributions (coronary vessels and valves). The UCS also repressed the transgene in the aorta, outlet septum, posterior cardiac plexus, cardiac and extra-cardiac nerves and neural ganglia. We used homologous recombination and conditional deletion to generate three new alleles in which the *Bmp2* 3'UTR was altered as follows: a UCS flanked by *loxP* sites with or without a neomycin resistance targeting vector, or a deleted UCS. Deletion of the UCS was associated with elevated *Bmp2* mRNA and BMP signaling levels, reduced fitness, and embryonic malformations.

Keywords

neural; birth defects; genetics; transcription; mutagenesis; mammal

INTRODUCTION

BMP2 is a critical signal whose concentration influences essential morphogenetic mechanisms in multiple structures. As expected for a morphogen, precise regulation of BMP2 levels and downstream signaling is crucial. BMP2-deficiencies lead to embryo

Correspondence: Melissa B. Rogers, Ph.D., Microbiology, Biochemistry & Molecular Genetics, Rutgers - NJ Medical School, Bldg. UH Cancer Center, Room F1216, 185 South Orange Ave., Newark, NJ 07103, Phone 973 972 2984, Fax 973 972 1865, rogersmb@njms.rutgers.edu.

*equal author contributions

lethality as early as E7.5 to E9 with defects in both extraembryonic and embryonic tissues (Ma *et al.*, 2005; Singh *et al.*, 2008; Zhang and Bradley, 1996). The absence of BMP2 disrupts key morphological processes such as enclosure of the fetus in extraembryonic tissues and the positioning of ventral structures such as the gut endoderm and heart (Gavrilov and Lacy, 2013), heart specification and cardiogenesis (Ma *et al.*, 2005; Rivera-Feliciano and Tabin, 2006; Zhang and Bradley, 1996), primordial germ cell generation (Ying and Zhao, 2001), and craniofacial development (Graf *et al.*, 2016). BMP signal gradients control the dorsal-ventral patterning and morphogenesis of the neural tube with BMP2 playing irreplaceable roles in neural plate morphogenesis (Ybot-Gonzalez *et al.*, 2007) and cranial neural tube closure and neural crest cell migration (Castranio and Mishina, 2009; Correia *et al.*, 2007; Kanzler *et al.*, 2000). In the adult, BMP2 is required to maintain bone function and quality post-weaning (Tsuji *et al.*, 2006; Yang *et al.*, 2013) and for the uterine decidual response during embryo implantation (Lee *et al.*, 2007).

Sub-normal levels of BMP2 also negatively impact embryogenesis. Later studies of mice heterozygous for global ablation of *Bmp2* (Zhang and Bradley, 1996) identified cephalic neural tube defects in heterozygotes (Uchimura *et al.*, 2009). Detailed analyses of compound mutant mice heterozygous for a *Bmp2* null allele with a *Bmp4* null or hypomorphic allele identified embryonic and post-natal lethality associated with multiple organ defects (Goldman *et al.*, 2009; Uchimura *et al.*, 2009). In the limb, a threshold level of BMP signaling resulting from the combined activities of BMP2, 4, and 7 is required for normal osteogenesis (Bandyopadhyay *et al.*, 2006). Consistent with the absolute requirement for BMP2 in decidualization, a *Bmp2* hypomorphic allele created by appending a Neo cassette to the *Bmp2* 3' UTR caused embryonic defects (Singh *et al.*, 2008). Dams with the hypomorphic allele exacerbated the embryonic phenotype. These studies showed that other BMP ligands such as BMP4 and BMP7 that are synthesized in overlapping or adjacent patterns fail to fully compensate for insufficient levels of maternal and embryonic BMP2.

Although multiple strategies have demonstrated the detrimental impact of elevated BMP signaling (Estrada *et al.*, 2011; Galvin *et al.*, 2000; Komatsu *et al.*, 2013; Singh *et al.*, 2008; Tylzanowski *et al.*, 2006), few studies have directly assessed the impact of elevated BMP2 levels. The apparent failure of embryos synthesizing elevated BMP2 in hair follicles provides circumstantial evidence that elevated BMP2 can be embryo-lethal (Blessing *et al.*, 1993). In contrast, mice that over expressed *Bmp2* in vascular smooth muscle cells survived with no reported developmental abnormalities. These mice had enhanced atherosclerotic intimal calcification in the *ApoE* null/high cholesterol model (Nakagawa *et al.*, 2010). Our analyses of new regulatory alleles that stimulate BMP2 synthesis begin to fill this gap in our understanding of how excess BMP2 impacts development.

Post-transcriptional gene regulation mechanisms involving the 3' untranslated region (UTR) often control the rapid and precise synthesis of potent signaling proteins like BMP2. *Bmp2* gene expression is greatly influenced by post-transcriptional mechanisms (reviewed in (Rogers *et al.*, 2015)). The entire *Bmp2* 3' UTR is extensively conserved between chicken and mammals (Fritz *et al.*, 2004; Hu *et al.*, 2006; Tian *et al.*, 2005). In addition, a unique ultra-conserved sequence (UCS) of several hundred nucleotides in the 3' UTR is 73% identical between mammals and fishes with some motifs present in the *AmphiBMP2/4* gene

in *Amphioxus*, a chordate cousin whose family branched 650 million years ago (Abrams *et al.*, 2004; Fritz *et al.*, 2006; Fritz *et al.*, 2004). This remarkable evolutionary conservation strongly supports ancient regulatory mechanisms necessary for controlling BMP2 synthesis during critical periods such as embryogenesis.

The UCS from both mice and humans can repress diverse gene constructs in developing and adult cells *in vitro* and *in vivo* (Devaney *et al.*, 2009; Fotinos *et al.*, 2016; Fotinos *et al.*, 2014; Jiang *et al.*, 2010; Kruithof *et al.*, 2011a; Kruithof *et al.*, 2011b). It is important to emphasize that the UCS does not repress in ALL cell types. Indeed, the UCS activates transcription in retinoic acid treated F9 embryonal cells and transformed lung cells (Abrams *et al.*, 2004; Fritz *et al.*, 2004; Jiang *et al.*, 2010). Furthermore, post-transcriptional control only works after a gene is transcribed. In embryos and many non-transformed tissues, the *Bmp2* gene appears to be “active”; *i.e.*, transcribed in specific tissues, but a post-transcriptional block limits BMP2 synthesis in a subset of those tissues. In these cells, reduced levels of post-transcriptional repression may lead to ectopic, precocious, and/or elevated levels of this key instructive developmental signal.

The purpose of this study was to use gene targeting in mice to assess the effects of deleting the UCS in the 3' UTR of the BMP2 mRNA on viability, morphology, and development. Three new *Bmp2* alleles were generated: a conditional allele with the UCS flanked by *loxP* sites (*Bmp2^{flUCS}*), a conditional allele with the UCS and a *Pgk-neo* targeting vector flanked by *loxP* sites (*Bmp2^{UCSNeo}*), and an allele with the UCS deleted (*Bmp2^{UCS}*). Our findings confirm that the UCS limits *Bmp2* mRNA abundance and BMP signaling *in vivo* and that disturbing *Bmp2* 3' UTR-mediated events negatively impact embryonic development.

RESULTS

Modified *Bmp2* 3'UTRs reduce survival

Given the unchanged nature of the *Bmp2* UCS over evolutionary time, we hypothesized that its deletion would decrease viability in mammals. To test this hypothesis, we used a new floxed UCS *Bmp2* allele in which the repressive UCS within the 3' UTR is flanked with *loxP* sites at +22 and +714 relative to the stop codon (*Bmp2^{flUCS}*, MMRRC# 042043, Fig. 1). Mice with one or two copies of the floxed allele (*Bmp2^{flUCS/+}* or *Bmp2^{flUCS/flUCS}*) were viable and fertile. Mice bearing the floxed UCS allele were bred to mice bearing a constitutively expressed Cre gene under the transcriptional control of a human cytomegalovirus promoter (Jackson Labs #B6.C-Tg(CMV-cre)1Cgn/J). The CMV-Cre gene is constitutively expressed from early embryogenesis on and reliably mediates the global recombination of a floxed gene in all tissues. The resulting pups had one copy of the recombined *Bmp2* allele (*Bmp2^{UCS/+}*) without the UCS in all tissues.

To test the impact of the complete absence of the UCS, we bred mice with 1 copy each of the recombined allele (*Bmp2^{UCS/+}*) and *CMV-Cre* to mice with 2 floxed alleles (*Bmp2^{flUCS/flUCS}*). Among the pups derived from this mating, 50% were expected to inherit the CMV-Cre gene. The floxed UCS allele in these pups would sustain a deletion of the UCS. The expected proportion of each genotype were 25% each *Bmp2^{flUCS/+}* and *Bmp2^{UCS/flUCS}* without *CMV-Cre* and 25% each of *Bmp2^{UCS/+}* or *Bmp2^{UCS/UCS}* with

CMV-Cre. We tabulated the number of pups with each genotype at weaning. Table 1 shows that mice with all potential genotypes: one copy of the floxed allele (*Bmp2^{fl/+}*), 1 copy of the recombined allele mice (*Bmp2^{UCS/+}*), compound heterozygotes bearing 1 copy each of the floxed and the recombined alleles (*Bmp2^{flUCS/fl}*), or homozygotes for the recombined *Bmp2* gene (*Bmp2^{UCS/UCS}*) were viable. However, *Bmp2^{UCS}* homozygotes were significantly underrepresented (Chi squared equals 25.5 with 3 degrees of freedom; 2 tailed P value < 0.0001). A few pups were found dead between birth and weaning, but the frequency did not differ from that typically observed in our colony, nor was any particular genotype over-represented.

BMP signaling and BMP2 in particular are required for normal uterine function during pregnancy (Clementi *et al.*, 2013; Lee *et al.*, 2007; Nagashima *et al.*, 2013). Therefore, we assessed the impact of parental homozygosity for the floxed alleles (*Bmp2^{flUCS/flUCS}*) or parental heterozygosity for the recombined allele (*Bmp2^{UCS/+}*) on viability. The genotype of the parents did not alter the underrepresentation of pups homozygous for the UCS deletion (Table 1). The genotypes of pups derived from mothers or fathers with either allele combination were both significantly skewed from Mendelian expectations (maternal *Bmp2^{flUCS/flUCS}*; Chi squared equals 19.5 with 3 degrees of freedom; 2 tailed P value < 0.0001; paternal *Bmp2^{flUCS/flUCS}*; Chi squared equals 11.8 with 3 degrees of freedom; 2 tailed P value < 0.0002). These results do not support a large maternal influence of either having two floxed alleles or one allele lacking the UCS.

The floxed *Bmp2* allele was generated *via* an allele bearing a 1.9 kb *Pgk-neo* cassette (see Methods). Mice with one or two copies of the Neo-containing allele inserted into the *Bmp2* 3' UTR (*Bmp2^{UCSNeo/+}* or *Bmp2^{UCSNeo/Neo}*, MMRRC #42279, Fig. 1C) also were viable and fertile. However, a previous study showed that compound heterozygotes bearing a deletion of the *Bmp2* coding region and an allele that generates the same Neo cassette appended to the end of the *Bmp2* transcript impacted embryonic survival (Singh *et al.*, 2008). Therefore, we quantified the impact of the *Pgk-neo* cassette on mice lacking the UCS. We bred mice with 1 copy each of the recombined allele (*Bmp2^{UCS/+}*) and *CMV-Cre* to mice with 2 Neo-bearing alleles (*Bmp2^{UCSNeo/UCSNeo}*). Table 2 shows that mice with one copy of the *Bmp2^{UCSNeo}* allele or the recombined *Bmp2^{UCS}* allele formed a majority of the surviving pups. As observed in the progeny of parents bearing the floxed allele, pups with two recombined alleles (*Bmp2^{UCS/UCS}*) were significantly underrepresented.

Furthermore, compound heterozygotes bearing 1 copy each of the *Bmp2^{UCSNeo}* allele and the recombined *Bmp2^{UCS}* allele were significantly underrepresented. Again, the resulting genotypes were significantly skewed from Mendelian expectations (Chi squared equals 102.5 with 3 degrees of freedom; 2 tailed P value < 0.0001). As with the floxed alleles, we failed to observe an obvious impact of parental genotype with the distribution of pup genotypes being equally skewed from expectations (maternal *Bmp2^{UCSNeo/UCSNeo}*; Chi squared equals 31.4 with 3 degrees of freedom; 2 tailed P value < 0.0001; paternal *Bmp2^{UCSNeo/UCSNeo}*; Chi squared equals 30.5 with 3 degrees of freedom; 2 tailed P value < 0.0001). These observations suggest that both the insertion of the 1.9 kb of targeting cassette in the middle of the 3' UTR and the deletion of the UCS severely impact tissues sensitive to changes in *Bmp2* regulation.

Previously, we had never observed developmental anomalies in many litters of embryos bearing the ubiquitously expressed CMV-Cre gene (Kruithof *et al.*, 2011a; Kruithof *et al.*, 2011b). However, tissue-selective expression of Cre-recombinase has been reported to cause developmental anomalies (Schmidt-Suprian and Rajewsky, 2007; Wang *et al.*, 2015). Therefore, we assessed the impact of the UCS deletion in mice lacking Cre-recombinase. Parents with 1 copy each of the recombined allele (*Bmp2*^{UCS/+}) alone were bred. The expected proportions were 25% wild type, 50% heterozygous, and 25% homozygous UCS null pups. Table 3 shows that mice homozygous for the recombined *Bmp2* gene (*Bmp2*^{UCS/UCS}) were significantly underrepresented (Chi squared equals 6.663 with 2 degrees of freedom, two-tailed P value < 0.036). The deletion appeared to more severely impact female pups.

UCS deletion increased *Bmp2* mRNA abundance and BMP signaling

The UCS repressed diverse genes bearing different promoters and 3'UTRs in many cultured cell types and mouse tissues (Devaney *et al.*, 2009; Fotinos *et al.*, 2016; Fotinos *et al.*, 2014; Jiang *et al.*, 2010; Kruithof *et al.*, 2011a; Kruithof *et al.*, 2011b). Therefore, we hypothesized that Cre-mediated deletion of the UCS will induce elevated or ectopic BMP2 synthesis. We used reverse transcription and real time PCR to determine the effect of UCS deletion on *Bmp2* mRNA expression in embryos obtained from dams bred as described in Tables 1 – 3. Because we were unable to authenticate antibodies directed at BMP2 specifically, we assessed BMP signaling using western blotting and authenticated antibodies directed at phosphorylated, BMP-activated SMAD proteins. *Bmp2* mRNA levels and the phosphorylation of SMAD1/5/9 (pSMAD1/5/9, HGNC:6774) both were significantly elevated in E10.5 embryos lacking the UCS relative to embryos with 1 wild type and one floxed allele (Fig. 2A, B). Furthermore, embryos heterozygous for the UCS deletion (*Bmp2*^{UCS/+} or *Bmp2*^{UCS/FL}) displayed intermediate levels of *Bmp2* mRNA abundance and BMP signaling.

Among many litters of embryos, we observed congenitally deformed embryos and embryos undergoing resorption that likely account for the reduced proportion of specific genotypes at birth. We tabulated the frequency and nature of defects at E10.5. Each of the 3 breeding strategies generated embryonic phenotypes that were highly variable as described below, with no parental combination obviously promoting any particular defect. Thirty out of 65 photographed and inspected embryos that were heterozygous for the UCS deletion had observable defects (46%). Twenty-six out of 43 homozygous embryos had observable defects (60%). In addition, at E10.5, we observed uterine swellings that contained apparently reabsorbing embryos that could not be cleanly removed and genotyped. Defects ranged from severely deformed, runted, fragile embryos to distinct neural tube defects (NTDs) and ventral body wall defects to subtler defects such as edema and superficial hemorrhage. Samples of each are shown in Fig. 3. NTDs were most commonly observed, although embryos with NTDs often had other defects such as delays ascertained by limb bud development. Fig. 3 shows representative embryos with open and closed neural tube and ventral body wall defects (E9.5, E10.5, 11.5) and holoprocencephaly (inadequate midline division of forebrain), anophthalmia (absence of one or both eyes), and micrognathia

(undersized jaw; E13.5). The presence of edema in some embryos is consistent with reduced cardiac function (Fig. 3B, lower left panel).

Homozygous deletion of the UCS was not uniformly embryo-lethal as apparently normal embryos were observed (Fig. 3B) and healthy pups were born (Tables 1–3). Defective embryos heterozygous for the deletion and one wild type or floxed allele also were observed (Fig. 3A, C). UCS-mediated post-transcriptional repression is just one of several mechanisms that repress *Bmp2* gene expression in particular (Rogers *et al.*, 2015) and BMP signaling in general (Katagiri and Watabe, 2016). Other regulators may partly compensate for deletion of the repressive UCS. In the mixed strain 129 and C57Bl/6 background used here, genetic variation may influence the efficacy of alternative repression mechanisms. Indeed, different inbred strains were associated with variation in the frequency and severity of defects observed in the survival of *Bmp2* null embryos and in *Bmp2* chimeras of *Bmp2* null ES and WT cells (Castranio and Mishina, 2009; Goldman *et al.*, 2009; Uchimura *et al.*, 2009). We observed that the level of SMAD1/5/9(8) phosphorylation in embryos with visible anomalies or growth retardation was nearly three times that of embryos with apparently normal morphologies (Fig. 3E, $n = 37$ defective vs. 23 normal E10.5 embryos, $p < 2 \times 10^{-6}$). Elevated BMP signaling was observed in all defective embryos including those with both the *Bmp2*^{UCS^{Neo} and the *Bmp2*^{UCS} alleles.}

DISCUSSION

The new alleles described here query the impact of UCS - mediated regulation in the context of normal transcriptional regulation. In contrast, we previously assessed UCS function using a *lacZ* reporter gene bearing 3.9 kb of *Bmp2* non-coding sequence that included the distal promoter and the intact 3'UTR. The intact reporter was expressed in several tissues that synthesize BMP2: pericardial mesoderm, interdigital cells, eye, cartilage primordium, and trigeminal nerves (Kruithof *et al.*, 2011b). Deletion of the UCS both intensified and expanded the domains of β gal activity into cells adjacent to regions that expressed the intact transgene. Additional ectopic expression was observed developing neural and cardiovascular tissues. Assuming the strictly post-transcriptional role for the UCS supported by *in vitro* findings (Fotinos *et al.*, 2016; Fotinos *et al.*, 2014; Jiang *et al.*, 2010; Kruithof *et al.*, 2011a), reporter gene transcription must have occurred in the cells with the expanded β gal activity.

A shortcoming of the previous studies was that the relatively small reporter gene did not completely recapitulate endogenous BMP2 transcription patterns in all tissues. For example, the reporter gene was not expressed in the roof plate of the telencephalon or the dorsal surface ectoderm, where BMP2 plays a key role in cephalic neural tube closure (Castranio and Mishina, 2009; Furuta *et al.*, 1997). Similarly, reporter gene activity was undetectable in the craniofacial structures where BMP2 influences cranial neural crest-derived bone development (Bonilla-Claudio *et al.*, 2012). These limitations were likely due to the absence of distant *Bmp2* regulatory regions and those in the proximal promoter and introns (Rogers *et al.*, 2015). In contrast, the new *Bmp2* alleles bear 3'UTR modifications of the endogenous gene. All regulatory elements outside of the *loxP*-flanked UCS are intact and should drive transcription in all appropriate tissues.

Both *in vivo* and *in vitro* observations support a widely spread repressive role for the UCS (Fotinos *et al.*, 2016; Fotinos *et al.*, 2014; Jiang *et al.*, 2010; Kruithof *et al.*, 2011a; Kruithof *et al.*, 2011b; Rogers *et al.*, 2015). Consistent with these studies, *Bmp2* mRNA abundance and BMP signaling was inversely proportional to the number of UCS copies in mid-gestation embryos (Fig. 2A, B). Numerous studies have shown that the 3'UTR of mRNAs regulates gene expression at a post transcriptional level either by regulating mRNA stability and thus RNA abundance or translation efficiency or both. The observation that UCS deletion elevates *Bmp2* RNA abundance is consistent with a role in controlling *Bmp2* mRNA stability. Ongoing studies are investigating the *trans*-regulatory factors that may control *Bmp2* expression via the UCS.

The positive correlation between the *Bmp2*^{UCS} allele and deformed embryos is consistent with our hypothesis that the UCS restrains BMP2 synthesis. We initially hypothesized that the *Bmp2*^{UCSNeo} allele might have produced lower BMP2 levels based upon the hypomorphic effect of an allele with the same *Pgk-neo* cassette appended to the transcript (Singh *et al.*, 2008). However, our results are not consistent with reduced BMP2 levels in embryos with the *Bmp2*^{UCSNeo} allele. Indeed, the range of phosphorylated SMAD1/5/9(8) levels in embryos with one copy of the Neo-containing allele (*Bmp2*^{UCSNeo/+}) or compound heterozygotes with 1 copy each of the Neo and the recombined allele (*Bmp2*^{UCSNeo/UCS}) were comparable to embryos with other genotypes (Fig. 3E).

Two points are relevant to this observation. First, the structure of a transcript with a 1.9 kb Neo cassette inserted within the 3'UTR (our allele) may differ greatly from a transcript bearing the same cassette appended to the end as in Singh *et al.* Our understanding of how RNA structure *per se* impacts post-transcriptional processes is limited. Therefore, the impact of each alteration must be defined empirically. Second, Fig. 3E shows average pSMAD1/5/9(8) levels in embryos with the *Bmp2*^{UCS/UCSNeo} genotype were not significantly different from those with the *Bmp2*^{UCS/UCS} genotype. Elevated pSMAD levels in the overtly defective embryos account for the upward spread of pSMAD level in both genotypes. Our working hypothesis is that both the Neo insertion and the deletion of the UCS disrupt the precision of post-transcriptional *Bmp2* regulatory processes leading to a greater range of BMP2 levels. Redundant layers of gene expression and signaling control can compensate for some added variation, but only partly.

Our analysis of morphological defects found in the embryos showed neural tube and ventral body wall defects to be among the most common defects observed (Fig. 3). Such ventral and dorsal patterning defects also are prevalent in mice with reduced BMP2 levels (Castranio and Mishina, 2009; Correia *et al.*, 2007; Gavrillov and Lacy, 2013; Graf *et al.*, 2016; Kanzler *et al.*, 2000; Ma *et al.*, 2005; Rivera-Feliciano and Tabin, 2006; Ybot-Gonzalez *et al.*, 2007; Ying and Zhao, 2001; Zhang and Bradley, 1996). Thus, as expected for a true morphogen, too little or too much of this ligand disrupts morphogenesis.

Despite strong evolutionary pressure against sequence change in the UCS (Abrams *et al.*, 2004; Fritz *et al.*, 2004), some individuals survive the absence of the UCS and develop to adulthood without obvious anomalies. The simplest explanation is that excess BMP2 levels activate other repressors that dampen BMP signaling. Indeed, BMP signaling directly

induces the extracellular antagonist NOGGIN, the intracellular repressor SMAD6, and the miR-17 family of miRNAs which repress BMPRII (Gazzerro and Minetti, 2007; Sun *et al.*, 2013). Negative feedback from excess BMP signaling would buffer the developing embryo from elevated BMP2 levels. Our observation that BMP signaling levels were significantly elevated in embryos with overt anomalies relative to those that were visibly normal (Fig. 2C) is consistent with the hypothesis that the UCS is just one of many redundant restraints on the activity of this potent growth factor. Just as different inbred strains vary in susceptibility to reduced BMP2 levels (Castranio and Mishina, 2009; Goldman *et al.*, 2009; Uchimura *et al.*, 2009), genetic variation in other BMP signaling repressors may compensate for deletion of the UCS.

The incomplete penetrance of these regulatory alleles is consistent with a reduction in the developmental robustness or “canalization” of these embryos. The UCS in the 3′UTR is a natural target of miRNA-mediated regulation that we have begun to explore (Fotinos *et al.*, 2016; Fotinos *et al.*, 2014). Interestingly, miRNAs have been proposed as part of a buffering network that ensures normal development in the face of genetic and environmental variation (Posadas and Carthew, 2014). Critical growth factors whose transcripts have conserved 3′UTRs like *Bmp2* are prime candidates for such a stabilizing mechanism.

The UCS was a repressive regulatory element in a majority of tested cell types including all mesenchymal cells and non-transformed lung cells (Fotinos *et al.*, 2016; Fotinos *et al.*, 2014; Jiang *et al.*, 2010; Kruithof *et al.*, 2011a; Kruithof *et al.*, 2011b; Rogers *et al.*, 2015). However, the UCS is an activator in retinoid-treated F9 embryonal cells, in transformed bronchial epithelial cells, and in human lung adenocarcinoma cells (Abrams *et al.*, 2004; Fritz *et al.*, 2004; Jiang *et al.*, 2010). All of these cells strongly transcribe *Bmp2*-driven reporter constructs and synthesize high levels of BMP2. Thus it is formally possible that deletion of the UCS also reduces BMP2 synthesis in some embryonic tissues. We did not observe reduced reporter gene expression in embryos bearing the short transgene (Kruithof *et al.*, 2011a; Kruithof *et al.*, 2011b). However, deletion of the UCS caused exuberant beta-galactosidase synthesis that would mask small regions where reduced reporter gene expression may have occurred. Furthermore, the small *lacZ* reporter lacked distantly located enhancers that would activate transcription (Rogers *et al.*, 2015).

The potential of the UCS to act as a switch that may activate or repress depending on cell type is highly relevant in the context of embryogenesis where *Bmp2* expression is highly dynamic. A recent study supports the idea that midline positioning of the dorsal mesentery depends on the relative, rather than the actual, amount of BMP signaling on the left and right sides of the developing embryo (Arraf *et al.*, 2016; Gavrilov and Lacy, 2016). By potentially up-regulating BMP2 synthesis in some tissues, but down-regulating in other tissues, the UCS deletion would disrupt the relative levels of BMP2 in developing tissues and profoundly influence tissue morphogenesis. An expanding body of evidence supports the critical need to maintain BMP2 levels within a narrow developmentally tolerated range. Regulatory mutations as in these new alleles are essential reagents for elucidating the role of gene expression in establishing an acceptable level of BMP2. Despite the experimental challenges, a full assessment of the mechanisms that control the quantity and pattern of

BMP2 and other important development factors is required to fully understand animal development.

METHODS

Generation of mice with *Bmp2* alleles with modified 3'UTRs

All animals were handled in accordance with the Guidelines for Care and Use of Experimental Animals and approved by the NJ Medical School Institutional Animal Care and Use Committee (IACUC protocol #15069). A *Bmp2* fragment was isolated from strain 129 and used to construct a 15.9 kb conditional gene targeting vector (Fig. 1A). The following fragments were subcloned into a bluescript SK vector in order: **1.** The upstream homology domain (uHOM) beginning at a Sall site 7313 bp upstream of the translational stop codon to 76 bp past the stop codon followed by a 124 bp *loxP* site which replaced a mouse specific PvuII site. **2.** The *Bmp2* UCS from +100 to +446 relative to the stop codon ending at an AccI site. **3.** A 1.9 kb *Pgk-neo* cassette flanked by FRT sites and bearing a downstream *loxP* site replaced the AccI site. The *neo* gene conferred kanamycin resistance in *E. coli* and G418 resistance in mammalian cells. **4.** The downstream homology domain beginning at the AccI site at +446 and extending 6197 bp downstream.

The positions of the probes used for Southern analysis are shown in Fig. 1A. The sizes of the restriction fragments detected by these probes in the wildtype allele are shown above the locus. The fragment sizes detected by these probes in the targeted allele are shown below the construct.

Linearized targeting vector was electroporated into AB2.2 ES cells (Lexicon Genetics, Woodlands, TX). G418-resistant clones were screened by Southern blot using the indicated external probes flanking the *Pgk-neo* insertion (Fig. 1B). Germline targeted mice were generated at the Transgenic/Knock-out core at UT Southwestern Medical Center (UTSW) in Dallas, TX) by injection into C57Bl/6 blastocysts and subsequently screened for agouti coat color. The resulted chimeras were bred to C57BL/6 females and F1 agouti offspring were genotyped by PCR as described below. Subsequently, mice heterozygous for floxedUCS allele with a *Pgk-neo* cassette (*Bmp2*^{UCSNeo/+}, MMRRC #42279) were bred with mice bearing a FLP recombinase gene driven by the constitutive ROSA promoter (JAX #007844; 129S4/SvJae-Gt(ROSA)26Sortm2(FLP*)Sor/J) to remove the *Pgk-neo* cassette. This yielded mice with a *loxP*-flanked UCS (*Bmp2*^{flUCS/+}, MMRRC# 042043). A ubiquitously expressed *CMV-Cre* gene under the transcriptional control of the cytomegalovirus promoter (JAX #006054; B6.C-Tg(CMV-cre)1Cgn/J) was then introduced into the *Bmp2*^{flUCS/+} mice to delete the UCS and to generate a recombined null UCS allele (*Bmp2*^{UCS/+}). The wildtype and 3 new alleles are shown in Fig. 1C.

These mice will be made available to the research community from the authors or the Mutant Mouse Resource & Research Centers (<https://www.mmrrc.org/>).

DNA isolation

Genomic DNA was isolated from tails from mouse pups at weaning and from either tails or amnions of embryos as follows. Tails or amnion membranes were lysed with DNA lysis

buffer (100 mM Tris, pH 8.5; 5 mM EDTA; 200 mM NaCl; 0.2% SDS; 0.2 mg/ml proteinase K added fresh) with rotation at 50 – 55°C for 12–16 hours. After solubilizing, debris was spun down for 10 minutes in a micro-centrifuge at full speed (16,000 × g). One volume of isopropanol was added to supernatants and mixed by inversion. DNA was centrifuged for 5 minutes at full speed, then washed with 70% ethanol.

Genotyping

Genomic DNA was genotyped by semi-quantitative PCR as follows: an initial denaturation at 97°C for 5 min; followed by 28 cycles of denaturation at 94°C for 30 secs; annealing at 55°C for 45 sec; and extension at 72°C for 1 min; ending with a final cycle of 94°C for 1 min, 55°C for 1 min, and 72°C for 5 min. Approximate PCR primer locations are shown in Fig. 1D. Primers A (5' GTC GTT AGC ACA GCA AGA ATA AA 3') and B (5' AAA GTG TTC ATT GGG AAA TAT TAA AGT 3') amplified a 121 bp fragment from the wildtype allele or a 245 bp fragment from the *Bmp2^{flUCS}* or *Bmp2^{UCSNeo}* alleles. Primers C (5' TCC AGC ACA TGA AGT ATA ATG GT 3') and D (5' GCA AGA AAC AGA CAG CAA GTG 3') amplified a 236 bp fragment from the wildtype allele or a 346 bp fragment from the *Bmp2^{flUCS}* allele. Primers E (5' GGG AGG ATT GGG AAG ACA ATA G3') and D amplified a 138 bp fragment from the *Bmp2^{UCSNeo}* allele. Primers A and D amplified a 509 bp fragment from the wildtype allele or a 246 bp fragment from the *Bmp2^{UCS}* allele. Fig. 1E shows a sample PCR.

Embryo fixation and histology preparation

Pregnant mice were euthanized and sacrificed according to IACUC regulations at embryonic stages E9.5, 10.5, and 13.5. Whole embryos were dissected in phosphate buffer solution (PBS) and separated from the amnion which was used for genotyping. Embryos were photographed and compared with littermates before fixation in chilled 3.7% paraformaldehyde-PBS solution or snap freezing for RNA and protein analyses.

RNA Isolation and Quantitative Real Time PCR (qPCR)

Total RNA was isolated from whole embryos using the RiboZol RNA isolation protocol (VWR Life Science, Radnor, PA, #N580) following manufacturer's instructions. RNA was quantified with a NanoDrop spectrophotometer (Agilent Technologies). RNA was reverse transcribed using the QuantiTect® Reverse Transcription kit (Qiagen Inc., Germantown, MD, # 205313) according to the manufacturer's instructions. Quantitative PCR was performed using the QuantiTect® SYBR® Green PCR kit (Qiagen Inc., Germantown, MD, #204145) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, #1855196). The mRNA expression was normalized to actin as the reference gene. Relative *Bmp2* mRNA expression was calculated using CFX96 Manager software (Bio-Rad Laboratories, Hercules, CA, # 1845000). Intron-spanning primers were used to eliminate amplicons generated from any contaminating genomic DNA. The primer sequences used were *Bmp2* - Forward (TAGATCTGTACCGCAGGCA) and Reverse (GTTCTCTCCACGGCTTCTTC) and Actin - Forward (CGCCACCAGTTCGCCATGGA) and reverse (TACAGCCCCGGGGAGCATCGT).

Western blotting

Lysates from whole embryos were prepared in RIPA buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10% (v/v) Glycerol, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% Sodium deoxycholate, 2 mM EDTA pH 8.0, 1 mM PMSF, 1 mM aprotinin, and 20 μ M leupeptin). Equal amounts of protein determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, # 5000006) was separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane (45 μ M, Bio-Rad Laboratories, Hercules, CA, #1620115) at 90V for 90 minutes in a wet transfer apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were blocked with 5% milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) with 1% (v/v) Tween-20 (TBS-T) for 1 hour at room temperature and then incubated overnight at 4°C with the specific primary antibody in TBS-T with 3% (w/v) BSA. BMP signaling was measured using a polyclonal phospho-SMAD 1/5/9(8) antibody (Cell Signaling Technology, Danvers, MA, #9511) or a monoclonal phospho-SMAD 1/5/9 antibody (Cell Signaling Technology, Danvers, MA, #13820) at a dilution of 1:1000. Polyclonal total SMAD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, #sc-6031-R) and Actin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, #sc-1616-R) were used as loading controls at a dilution of 1:1,000 and 1:500 respectively. The pSMAD antibodies were authenticated using MC3T3-E1 (clone 4) and C3H 10T $\frac{1}{2}$ cells transfected with a *Bmp2* expression vector or a control vector expressing luciferase. Specific proteins were detected using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, # 1705061) and imaged using FluoroChem M (Protein Simple, San Jose, California). Densitometric analysis of the blots was done using AlphaView SA 3.0 software (Protein Simple, San Jose, California).

Acknowledgments

Grant support: Rutgers NJMS Dean's Biomedical Research Support Program; National Heart, Lung, and Blood Institute 1R01HL114751

References

- Abrams KL, Xu J, Nativelle-Serpentini C, Dabirshahsahebi S, Rogers MB. An evolutionary and molecular analysis of *Bmp2* expression. *J Biol Chem*. 2004; 279:15916–15928. [PubMed: 14757762]
- Arraf AA, Yelin R, Reshef I, Kispert A, Schultheiss TM. Establishment of the Visceral Embryonic Midline Is a Dynamic Process that Requires Bilaterally Symmetric BMP Signaling. *Dev Cell*. 2016; 37:571–580. [PubMed: 27326934]
- Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ. Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet*. 2006; 2:e216. [PubMed: 17194222]
- Blessing M, Nanney LB, King LE, Jones CM, Hogan BL. Transgenic mice as a model to study the role of TGF-beta-related molecules in hair follicles. *Genes Dev*. 1993; 7:204–215. [PubMed: 8436293]
- Bonilla-Claudio M, Wang J, Bai Y, Klysik E, Selever J, Martin JF. *Bmp* signaling regulates a dose-dependent transcriptional program to control facial skeletal development. *Development*. 2012; 139:709–719. [PubMed: 22219353]
- Castranio T, Mishina Y. *Bmp2* is required for cephalic neural tube closure in the mouse. *Dev Dyn*. 2009; 238:110–122. [PubMed: 19097048]
- Clementi C, Tripurani SK, Large MJ, Edson MA, Creighton CJ, Hawkins SM, Kovanci E, Kaartinen V, Lydon JP, Pangas SA, DeMayo FJ, Matzuk MM. Activin-like kinase 2 functions in peri-

- implantation uterine signaling in mice and humans. *PLoS Genet.* 2013; 9:e1003863. [PubMed: 24244176]
- Correia AC, Costa M, Moraes F, Bom J, Novoa A, Mallo M. Bmp2 is required for migration but not for induction of neural crest cells in the mouse. *Dev Dyn.* 2007; 236:2493–2501. [PubMed: 17676634]
- Devaney JM, Tosi LL, Fritz DT, Gordish-Dressman HA, Jiang S, Orkunoglu-Suer FE, Gordon AH, Harmon BT, Thompson PD, Clarkson PM, Angelopoulos TJ, Gordon PM, Moyna NM, Pescatello LS, Visich PS, Zoeller RF, Brandoli C, Hoffman EP, Rogers MB. Differences in fat and muscle mass associated with a functional human polymorphism in a post-transcriptional BMP2 gene regulatory element. *J Cell Biochem.* 2009; 107:1073–1082. [PubMed: 19492344]
- Estrada KD, Retting KN, Chin AM, Lyons KM. Smad6 is essential to limit BMP signaling during cartilage development. *J Bone Miner Res.* 2011; 26:2498–2510. [PubMed: 21681813]
- Fotinos A, Fritz DT, Lisica S, Liu Y, Rogers MB. Competing Repressive Factors Control Bone Morphogenetic Protein 2 (BMP2) in Mesenchymal Cells. *J Cell Biochem.* 2016; 117:439–447. [PubMed: 26212702]
- Fotinos A, Nagarajan N, Martins AS, Fritz DT, Garsetti D, Lee AT, Hong CC, Rogers MB. Bone morphogenetic protein-focused strategies to induce cytotoxicity in lung cancer cells. *Anticancer Res.* 2014; 34:2095–2104. [PubMed: 24778011]
- Fritz DT, Jiang S, Xu J, Rogers MB. A polymorphism in a conserved posttranscriptional regulatory motif alters bone morphogenetic protein 2 (BMP2) RNA:protein interactions. *Mol Endocrinol.* 2006; 20:1574–1586. [PubMed: 16497730]
- Fritz DT, Liu D, Xu J, Jiang S, Rogers MB. Conservation of Bmp2 post-transcriptional regulatory mechanisms. *J Biol Chem.* 2004; 279:48950–48958. [PubMed: 15358784]
- Furuta Y, Piston DW, Hogan BL. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development.* 1997; 124:2203–2212. [PubMed: 9187146]
- Galvin KM, Donovan MJ, Lynch CA, Meyer RI, Paul RJ, Lorenz JN, Fairchild-Huntress V, Dixon KL, Dunmore JH, Gimbrone MA Jr, Falb D, Huszar D. A role for smad6 in development and homeostasis of the cardiovascular system. *Nat Genet.* 2000; 24:171–174. [PubMed: 10655064]
- Gavrilov S, Lacy E. Genetic dissection of ventral folding morphogenesis in mouse: embryonic visceral endoderm-supplied BMP2 positions head and heart. *Curr Opin Genet Dev.* 2013; 23:461–469. [PubMed: 23706163]
- Gavrilov S, Lacy E. Symmetric BMPs on the Developmental Road. *Dev Cell.* 2016; 37:488–490. [PubMed: 27326926]
- Gazzerro E, Minetti C. Potential drug targets within bone morphogenetic protein signaling pathways. *Curr Opin Pharmacol.* 2007; 7:325–333. [PubMed: 17475557]
- Goldman DC, Donley N, Christian JL. Genetic interaction between Bmp2 and Bmp4 reveals shared functions during multiple aspects of mouse organogenesis. *Mech Dev.* 2009; 126:117–127. [PubMed: 19116164]
- Graf D, Malik Z, Hayano S, Mishina Y. Common mechanisms in development and disease: BMP signaling in craniofacial development. *Cytokine Growth Factor Rev.* 2016; 27:129–139. [PubMed: 26747371]
- Hu, J., Fritz, DT., Tian, B., Rogers, MB. Using Emerging Genome Data to Identify Conserved Bone Morphogenetic Protein (Bmp)2 Gene Expression Mechanisms. *ACM First International Workshop on Text Mining in Bioinformatics (TMBIO2006) Proceedings*; Arlington, VA, USA. New York, NY: ACM Press; 2006.
- Jiang S, Fritz DT, Rogers MB. A conserved post-transcriptional BMP2 switch in lung cells. *J Cell Biochem.* 2010; 110:509–521. [PubMed: 20432245]
- Kanzler B, Foreman RK, Labosky PA, Mallo M. BMP signaling is essential for development of skeletogenic and neurogenic cranial neural crest. *Development.* 2000; 127:1095–1104. [PubMed: 10662648]
- Katagiri T, Watabe T. Bone Morphogenetic Proteins. *Cold Spring Harb Perspect Biol.* 2016;8.
- Komatsu Y, Yu PB, Kamiya N, Pan H, Fukuda T, Scott GJ, Ray MK, Yamamura K, Mishina Y. Augmentation of Smad-dependent BMP signaling in neural crest cells causes craniosynostosis in mice. *J Bone Miner Res.* 2013; 28:1422–1433. [PubMed: 23281127]

- Kruithof BP, Fritz DT, Liu Y, Garsetti DE, Frank DB, Pregizer SK, Gaussin V, Mortlock DP, Rogers MB. An autonomous BMP2 regulatory element in mesenchymal cells. *J Cell Biochem.* 2011a; 112:666–674. [PubMed: 21268088]
- Kruithof BP, Xu J, Fritz DT, Cabral CS, Gaussin V, Rogers MB. An in vivo map of bone morphogenetic protein 2 post-transcriptional repression in the heart. *Genesis.* 2011b; 49:841–850. [PubMed: 21504044]
- Lee KY, Jeong JW, Wang J, Ma L, Martin JF, Tsai SY, Lydon JP, DeMayo FJ. Bmp2 is critical for the murine uterine decidual response. *Mol Cell Biol.* 2007; 27:5468–5478. [PubMed: 17515606]
- Ma L, Lu MF, Schwartz RJ, Martin JF. Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development.* 2005; 132:5601–5611. [PubMed: 16314491]
- Nagashima T, Li Q, Clementi C, Lydon JP, DeMayo FJ, Matzuk MM. BMPR2 is required for postimplantation uterine function and pregnancy maintenance. *J Clin Invest.* 2013; 123:2539–2550. [PubMed: 23676498]
- Nakagawa Y, Ikeda K, Akakabe Y, Koide M, Uraoka M, Yutaka KT, Kurimoto-Nakano R, Takahashi T, Matoba S, Yamada H, Okigaki M, Matsubara H. Paracrine osteogenic signals via bone morphogenetic protein-2 accelerate the atherosclerotic intimal calcification in vivo. *Arterioscler Thromb Vasc Biol.* 2010; 30:1908–1915. [PubMed: 20651281]
- Posadas DM, Carthew RW. MicroRNAs and their roles in developmental canalization. *Curr Opin Genet Dev.* 2014; 27:1–6. [PubMed: 24791686]
- Rivera-Feliciano J, Tabin CJ. Bmp2 instructs cardiac progenitors to form the heart-valve-inducing field. *Dev Biol.* 2006; 295:580–588. [PubMed: 16730346]
- Rogers MB, Shah TA, Shaikh NN. Turning Bone Morphogenetic Protein 2 (BMP2) on and off in Mesenchymal Cells. *J Cell Biochem.* 2015; 116:2127–2138. [PubMed: 25776852]
- Schmidt-Supprian M, Rajewsky K. Vagaries of conditional gene targeting. *Nat Immunol.* 2007; 8:665–668. [PubMed: 17579640]
- Singh AP, Castranio T, Scott G, Guo D, Harris MA, Ray M, Harris SE, Mishina Y. Influences of reduced expression of maternal bone morphogenetic protein 2 on mouse embryonic development. *Sex Dev.* 2008; 2:134–141. [PubMed: 18769073]
- Sun Q, Mao S, Li H, Zen K, Zhang CY, Li L. Role of miR-17 family in the negative feedback loop of bone morphogenetic protein signaling in neuron. *PLoS One.* 2013; 8:e83067. [PubMed: 24349434]
- Tian B, Hu J, Zhang H, Lutz CS. A large-scale analysis of mRNA polyadenylation of human and mouse genes. *Nucleic Acids Res.* 2005; 33:201–212. [PubMed: 15647503]
- Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nat Genet.* 2006; 38:1424–1429. [PubMed: 17099713]
- Tylzanowski P, Mebis L, Luyten FP. The Noggin null mouse phenotype is strain dependent and haploinsufficiency leads to skeletal defects. *Dev Dyn.* 2006; 235:1599–1607. [PubMed: 16598734]
- Uchimura T, Komatsu Y, Tanaka M, McCann KL, Mishina Y. Bmp2 and Bmp4 genetically interact to support multiple aspects of mouse development including functional heart development. *Genesis.* 2009; 47:374–384. [PubMed: 19391114]
- Wang L, Mishina Y, Liu F. Osterix-Cre transgene causes craniofacial bone development defect. *Calcif Tissue Int.* 2015; 96:129–137. [PubMed: 25550101]
- Yang W, Guo D, Harris MA, Cui Y, Gluhak-Heinrich J, Wu J, Chen XD, Skinner C, Nyman JS, Edwards JR, Mundy GR, Lichtler A, Kream BE, Rowe DW, Kalajzic I, David V, Quarles DL, Villareal D, Scott G, Ray M, Liu S, Martin JF, Mishina Y, Harris SE. Bmp2 in osteoblasts of periosteum and trabecular bone links bone formation to vascularization and mesenchymal stem cells. *J Cell Sci.* 2013; 126:4085–4098. [PubMed: 23843612]
- Ybot-Gonzalez P, Gaston-Massuet C, Girdler G, Klingensmith J, Arkell R, Greene ND, Copp AJ. Neural plate morphogenesis during mouse neurulation is regulated by antagonism of Bmp signalling. *Development.* 2007; 134:3203–3211. [PubMed: 17693602]
- Ying Y, Zhao GQ. Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. *Dev Biol.* 2001; 232:484–492. [PubMed: 11401407]

Zhang H, Bradley A. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Devl.* 1996; 122:2977–2986.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Targeting construct, alleles, and genotyping
A. A PvuII to an AccI fragment bearing the natural *Bmp2* UCS in exon 3 (+100 to +446 relative to the stop codon) was replaced by a 124 bp *loxP* site followed by the *Bmp2* UCS and 1.9 kb *Pgk-neo* cassette flanked by *FRT* sites and a downstream *loxP* site. The positions of 5' and 3' probes and restriction enzyme sites used for Southern analyses are indicated with the expected wildtype fragment sizes shown above and the targeted fragment sizes shown below the construct diagram. **B.** Genomic DNA from WT embryonic stem cells (lane 1) and correctly targeted cells (lane 2) were digested with the indicated enzymes and probes. **C.** A diagram of each allele and the positions of primers used for PCR genotyping. **D.** The expected size in bp of the amplicons generated by each primer pair by each allele. n.s. = not shown in E. **E.** Sample genotyping results.

Fig. 2. *Bmp2* mRNA expression and BMP signaling in embryos lacking the *Bmp2* UCS
A. *Bmp2* mRNA expression in E10.5 embryos normalized to actin determined using reverse transcription and real time PCR (Average $\pm$ SEM, n = 6–13). **B.** BMP signaling as assessed by normalizing phosphorylated SMAD1/5/9(8) (pSMAD) levels to actin levels in E10.5 embryos (Average $\pm$ SEM, n = 8–17). **C.** Western blot panels showing pSMAD1/5/9(8), total SMAD1/5/9(8) (tSMAD), and actin levels in representative pairs of E10.5 embryos.

Fig. 3. Malformations and BMP2 levels in embryos lacking the *Bmp2* UCS
Representative embryonic floxed or recombined heterozygotes (*Bmp2*^{fl/+} or *Bmp2*^{UCS/+}), compound heterozygotes (*Bmp2*^{fl/ UCS}), and recombined homozygotes (*Bmp2*^{UCS/ UCS}) lacking the UCS. **A.** E9.5, **B.** E10.5, **C.** E11.5, **D.** E13.5. Embryos within each panel are littermates. Both normal appearing (**N**) and defective (**D**) embryos with the same genotypes were observed. **E.** E10.5 embryos with elevated BMP signaling were more likely to be defective. The filled or open circles indicate the pSMAD1/5/9(8) levels of embryos that were visibly defective or visibly normal, respectively.

Table 1

Reduced fitness in mice lacking the *Bmp2* UCS

Mice with 1 copy each of the recombined allele (*Bmp2*^{UCS/+}) and *CMV-Cre* were bred to mice with 2 floxed alleles (*Bmp2*^{flUCS/flUCS}).

Expected genotypes:		25% <i>Bmp2</i> ^{+fl}	25% <i>CMV-Cre; Bmp2</i> ^{+fl} UCS	25% <i>Bmp2</i> ^{UCS/fl}	25% <i>CMV-Cre; Bmp2</i> ^{UCS/fl}	25% <i>CMV-Cre; Bmp2</i> ^{UCS/UCS}
Paternal genotype	Maternal genotype	Number of pups (%):				
<i>CMV-Cre; Bmp2</i> ^{UCS/+}	<i>Bmp2</i> ^{flUCS/flUCS}	35 (43%)	19 (23%)	20 (25%)	7 (9%)	
<i>Bmp2</i> ^{flUCS/flUCS}	<i>CMV-Cre; Bmp2</i> ^{UCS/+}	29 (29%)	34% (34%)	26 (26%)	11 (11%)	
All		64 (35%)	53 (29%)	46 (25%)	18 (10%)	

Reduced fitness in compound heterozygotes with a Neo insertion and a deleted *Bmp2* UCS

Table 2

Mice with 1 copy each of the recombined allele (*Bmp2* UCS^{+/+}) and *CMV-Cre* were bred to mice with 2 Neo alleles (*Bmp2*^{UCSNeo/UCSNeo}).

Expected genotypes:		25% <i>Bmp2</i> ^{+/UCSNeo}	25% <i>CMV-Cre; Bmp2</i> ^{+/ UCS}	25% <i>Bmp2</i> ^{UCS/UCSNeo}	25% <i>CMV-Cre; Bmp2</i> ^{UCS / UCS}
Paternal genotype	Maternal genotype	Number of pups (%):			
<i>CMV-Cre; Bmp2</i> UCS ^{+/+}	<i>Bmp2</i> ^{Neo/Neo}	43 (27%)	68 (42%)	31 (19%)	20 (12%)
<i>Bmp2</i> ^{Neo/Neo}	<i>CMV-Cre; Bmp2</i> UCS ^{+/+}	44 (23%)	79 (42%)	30 (16%)	19 (19%)
All		87 (25%)	147 (42%)	61 (17%)	56 (16%)

Table 3
Cre recombinase does not account for reduced fitness in mice lacking the Bmp2 UCS
Parents with 1 copy each of the recombined allele (*Bmp2 UCS^{+/+}*) were bred.

Expected genotypes:	25% <i>Bmp2^{+/+}</i>	50% <i>Bmp2^{+/ UCS}</i>	25% <i>Bmp2 UCS/ UCS</i>
Pup Sex	Number of pups (%):		
Male	44 (26%)	89 (52%)	37 (22%)
Female	41 (23%)	107 (60%)	31 (17%)
All	89 (25%)	202 (56%)	71 (20%)