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SPECIAL ISSUE REVIEWS–A PEER REVIEWED FORUM

Crosstalk Between Wnt and Bone Morphogenic Protein Signaling: A Turbulent Relationship

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The Wnt and the bone morphogenic protein (BMP) pathways are evolutionarily conserved and essentially independent signaling mechanisms, which, however, often regulate similar biological processes. Wnt and BMP signaling are functionally integrated in many biological processes, such as embryonic patterning in *Drosophila* and vertebrates, formation of kidney, limb, teeth and bones, maintenance of stem cells, and cancer progression. Detailed inspection of regulation in these and other tissues reveals that Wnt and BMP signaling are functionally integrated in four fundamentally different ways. The molecular mechanism evolved to mediate this integration can also be summarized in four different ways. However, a fundamental aspect of functional and mechanistic interaction between these pathways relies on tissue-specific mechanisms, which are often not conserved and cannot be extrapolated to other tissues. Integration of the two pathways contributes toward the sophisticated means necessary for creating the complexity of our bodies and the reliable and healthy function of its tissues and organs. *Developmental Dynamics 239:16–33, 2010.* © 2009 Wiley-Liss, Inc.

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INTRODUCTION

Cell-to-cell signaling undertakes a tremendous variety of biological functions during animal development and adult homeostasis. Remarkably, they are predominantly mediated by a small number of conserved molecular signaling pathways. The complexity of the response with required specificity can be brought about by a combination of multiple signal transduction pathways. Activity of a signaling pathway can influence that of the other, depending on the context, resulting in different cellular responses from the one achieved by just a single cascade. The effect may not only modify the degree of the outcome activity of the pathway but also cause qualitatively different biological effects. Together with intrinsic factors such as availability of cofactors and target genes, combinatorial activation of signaling pathways amplifies not only the magnitude but also the complexity of cellular response. This interaction is so-called "crosstalk" of multiple signaling pathways.

The Wnt and bone morphogenic protein (BMP) signaling pathways are implicated in many biological events such as stem cell maintenance, cell fate specifications, organogenesis, and carcinogenesis (Logan and Nusse, 2004; Moon et al., 2004; Varga and Wrana, 2005; Hardwick et al., 2008). Mechanisms of regulating each signal transduction pathway have been intensively studied (von Bubnoff and Cho, 2001; Derynck and Zhang, 2003; Gordon and Nusse, 2006; Huang and He, 2008). They are able to function independently from each other: by means of different ligands, different receptors and different cytoplasmic and nuclear signal transducers, without sharing any major pathway components. However, in many biological contexts Wnt and BMP ligands are expressed in overlapping or complementary manners, spatially or tempo-

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rarily, as if they are "crosstalking" to each other. In fact, recent studies have revealed many cases where these two pathways cooperate or attenuate each other, thus causing effects that cannot be achieved by either alone.

A difficulty in understanding the Wnt-BMP crosstalk is that the effect varies; it can either be synergistic or antagonistic, depending on the cellular context, as was first discovered in *Drosophila* development (Azpiazu et al., 1996; Carmena et al., 1998). One example of synergistic effects during vertebrate embryogenesis is seen in early *Xenopus* embryos, where Wnt8 and BMP4 are expressed in overlapping domains with both being required for induction of ventral mesoderm (Hoppler and Moon, 1998). They are not simply coexpressed or functioning redundantly: BMP signaling is indispensable for Wnt8 to exert its function. This is one of the first indications suggesting a synergistic effect of BMP and Wnt signals in vertebrates. In other contexts, Wnt and BMP signals have opposing functions. For instance, the cell fate of neural crest cells are biased to melanocyte by Wnt signals, while BMP signals induce neuron and glia, and repress melanogenesis (Jin et al., 2001). These two examples already clearly illustrate that context-dependent effects is a hallmark of crosstalk between these two signaling pathways, and suggest complexity in the molecular mechanism.

In this review, we analyze how crosstalk of Wnt and BMP pathways functions in different biological contexts by focusing on those embryonic tissues and those tumors in which the salient aspects of this interaction is best illustrated. We also explain several molecular mechanisms, which mediate the observed crosstalk between BMP and Wnt signaling.

SYNERGISTIC AND ANTAGONISTIC EFFECTS OF THE WNT AND BMP PATHWAYS IN DIFFERENT CONTEXTS

Patterning in the *Drosophila* Embryo

The power of genetics to analyze *Drosophila* development has been priceless for dissecting the signaling interactions between the Wnt (*wingless*) and BMP (*decapentaplegic*) pathways. In certain tissues, such as during *Drosophila* leg development, antagonism between these pathways is simply hardwired by mutual repression of each other's ligand-encoding gene, whereby Wnt signaling represses BMP expression and BMP signaling represses Wnt expression (e.g., Theisen et al., 1996).

The patterning of the mesoderm in *Drosophila* provides a perfect model system for investigating more complex, combinatorial signaling mechanisms between the Wnt and BMP signaling pathways. Here they involve synergy and antagonism in the same tissue and even in some of the same cells depending on the target gene (Fig. 1). Wnt and BMP signaling overlaps in the anterior domain of the segmental units of the *Drosophila* embryo (parasegments). The homeobox genes *bagpipe* and *even-skipped* read this positional information, but interpret it completely differently: while Wnt and BMP synergize to induce even-skipped expression (Carmena et al., 1998), Wnt signaling antagonizes BMP signaling to prevent bagpipe expression in the same domain (Azpiazu et al., 1996).

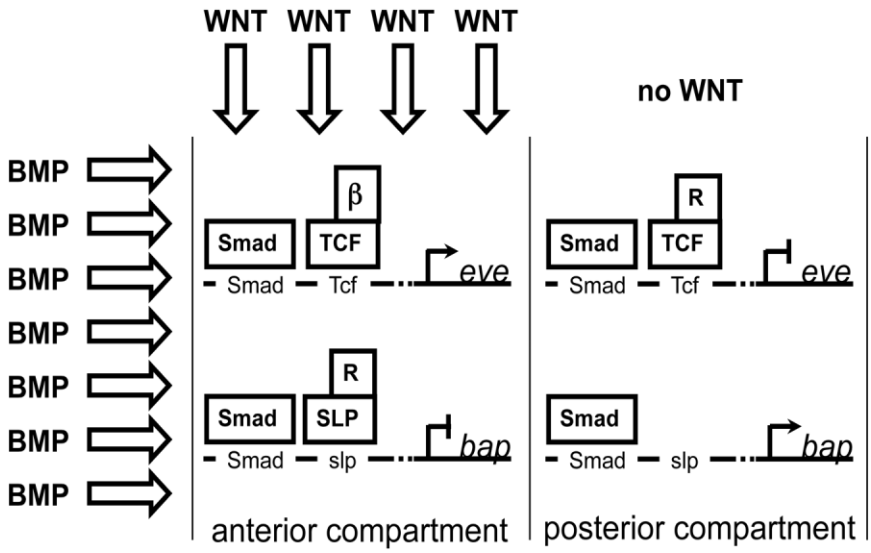

Fig. 1. Combinatorial Wnt and bone morphogenic protein (BMP) signaling regulates homeobox genes in the *Drosophila* mesoderm. Wnt and BMP signaling overlap in the anterior but not the posterior compartment of the embryonic segmental units in *Drosophila* embryos. The enhancer of the *even-skipped* gene (*eve*) integrates synergy between Wnt and BMP signaling through Smad and Tcf binding sites, which mediate activation of expression in the anterior compartment but repression by Tcf and a transcriptional corepressor (R, i.e., Groucho) in the posterior compartment where there is only BMP, but no Wnt signaling. The enhancer of the *bagpipe* (*bap*) gene mediates expression in a complementary pattern; in the absence of Wnt signaling it activates *bagpipe* expression through its Smad binding site; but it also contains a binding site for the FoxG-related transcription factor, a product of the *sloppy paired* (*slp*) gene, which associates with a transcriptional corepressor (R, i.e., Groucho) to repress *bagpipe* expression in the anterior compartment where Wnt signaling specifically induces *sloppy paired* (FoxG) expression. β indicates β-catenin/armadillo. Figure modified after Lee and Frasch (2005).

The Wnt and BMP signaling is differently integrated on the relevant enhancers of these genes; while the *even-skipped* enhancer has a BMP response element (Smad1/5/8 [*Mad*] and Smad4 [*Medea*] binding sites) next to a Wnt response element (Tcf [*pangolin*] binding sites; Knirr and Frasch, 2001) to mediate synergy; the *bagpipe* enhancer, to integrate antagonism, contains a BMP response element next to the binding site for a FoxG forkhead-family transcriptional repressor (Sloppy paired), which is up-regulated in these cells by Wnt signaling (Lee and Frasch, 2005) (Fig. 1).

Combinatorial Wnt (*wingless*) and BMP (*decapentaplegic*) signaling regulates development of the *Drosophila* midgut, and in particular homeotic gene expression in the endoderm (*labial*) and the associated visceral mesoderm (*Ultrabithorax*; Fig. 2A). This precise regulation of homeotic gene expression governs morphogenesis

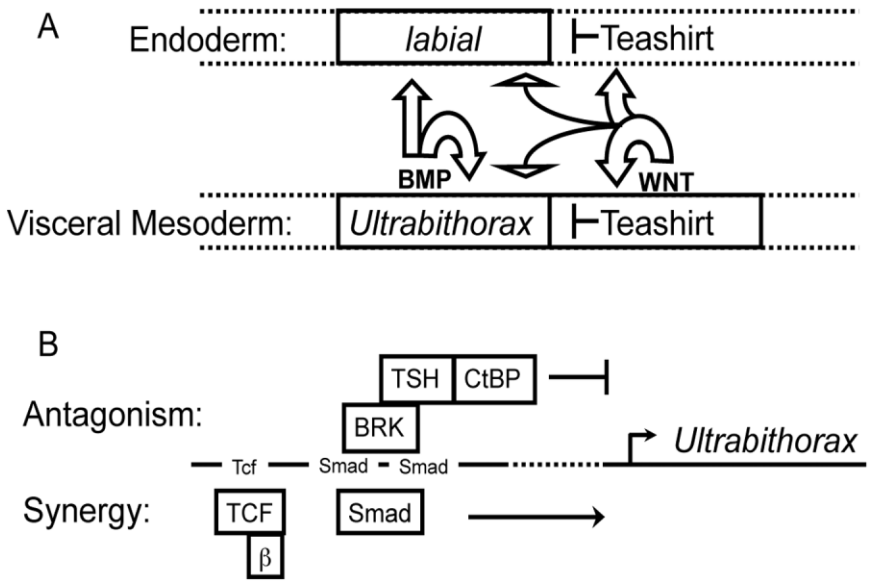

Fig. 2. Combinatorial Wnt and bone morphogenetic protein (BMP) signaling regulates homeotic genes in the *Drosophila* midgut. **A:** Extracellular Wnt and BMP signaling from the visceral mesoderm regulate expression of the homeotic genes *labial* in the endoderm and *Ultrabithorax* in the visceral mesoderm itself. At a distance from the Wnt expression domain, relatively low levels of Wnt signaling synergizes with BMP signaling to induce expression of *labial* and maintain *Ultrabithorax* expression; while close to the Wnt expression domain, higher levels of Wnt signaling antagonize BMP signaling by inducing expression of *Teashirt*, which encodes a transcriptional repressor that prevents expression of *labial* and *Ultrabithorax* in this domain. **B:** The *Ultrabithorax* enhancer integrates the synergistic and antagonistic regulation by Wnt and BMP signaling. A Wnt Response Element (WRE, containing conserved Tcf (pangolin) TCF binding sites) sits next to a BMP Response Element (BRE, containing Smad/MAD binding sites [SMAD]) to mediate synergy between BMP and relatively low levels of Wnt signaling. However, the BRE overlaps with binding sites for the Brinker sequence-specific DNA binding protein (BRK), which recruits Teashirt (TSH) and CtBP and thus forms a transcriptional repression complex on the BRE to antagonize BMP signaling in the domain with high Wnt signaling. β, β-catenin/armadillo. Figure modified after Saller et al. (2002).

and subsequent differentiation of specific cell types in the endoderm, such as in the labial expressing cells into the copper cells (e.g, Hoppler and Bienz, 1994). The Wnt ligand is expressed in the visceral mesoderm in a domain (parasegment 8) immediately posterior to the BMP ligand-expressing domain (parasegment 7). Autocrine BMP and low Wnt signaling synergize to maintain *Ultrabithorax* expression in the BMP expression domain; while just posteriorly, in the Wnt expression domain, high Wnt signaling antagonizes BMP signaling to repress *Ultrabithorax* expression (reviewed by Bienz, 1997). This regulation of *Ultrabithorax* in the visceral mesoderm layer is mirrored by *labial* regulation in the endoderm: BMP and low Wnt signaling synergize to induce *labial* expression in the endoderm next to the BMP expression domain in the visceral mesoderm; while further posterior, high Wnt signaling antagonizes BMP signaling-induced expression of *labial* adjacent to the Wnt expression domain (Hoppler and Bienz, 1995).

Synergy with low Wnt signaling is mediated by means of direct regulation by Tcf (*pangolin*) and β-catenin (*Armadillo*) function (Riese et al., 1997), while high Wnt signaling causes antagonism indirectly by means of up-regulation of *Teashirt* (Mathies et al., 1994; Waltzer et al., 2001), which encodes a Zinc-finger protein that assembles a transcriptional repressor complex containing the transcriptional corepressor CtBP and the sequence-specific DNA binding factor Brinker (Saller et al., 2002). The relevant enhancer region in *Ultrabithorax* integrates this intricate regulation perfectly (Fig. 2B). It contains a Wnt response element (Tcf [*pangolin*] binding site; Riese et al., 1997) next to a BMP response element (Smad1/5/8 [*Mad*] binding sites; Szuts et al., 1998) to mediate the observed synergy between BMP and low levels of Wnt signaling; but also several Brinker binding sites overlapping with this BMP response element to antagonize the positive regulation by BMP/Smad signaling of the *Ultrabithorax* enhancer directly by high levels of Wnt signaling (Saller et al., 2002).

Dorsal–Ventral Patterning of the Spinal Cord in Vertebrate Embryos

Dorsal–ventral patterning of the spinal cord in vertebrate embryos involves multiple signaling mechanisms (Helms and Johnson, 2003). The dorsal spinal cord is characterized by neural crest production and differentiation of dorsal interneurons, along with expression of genes such as *olig3* and *Math1* (Gowan et al., 2001; Takebayashi et al., 2002; Zechner et al., 2007). The dorsal spinal cord expresses Wnt (Wnt1, Wnt3a; Hollyday et al., 1995; Galli et al., 2007) and BMP (BMP2,4,7; Basler et al., 1993; Lee et al., 1998) ligands, both of which are involved in conferring generally dorsal-specific character in this region. Expression of BMP ligands is initiated by a contact with the surface ectoderm (Liem et al., 1995), and is responsible for Wnt1 expression (Burstyn-Cohen et al., 2004). Wnt signals enhance the BMP pathway as seen by an increase of phospho-smad1/5/8 and expression of downstream target gene *Msx1* (Ille et al., 2007). Overactivation of either pathway causes expansion of dorsal-specific domains (Liem et al., 1995; Timmer et al., 2002; Ille et al., 2007; Zechner et al., 2007; Alvarez-Medina et al., 2008), whereas loss-of-function of either results in a failure to specify dorsal-specific cell fates (Nguyen et al., 2000; Muroyama et al., 2002; Zechner et al., 2007), indicating that both Wnt and BMP signals are required for proper fate-specification at the dorsal neural tube. An important question concerns whether the two pathways are independently responsible for dorsal patterning: i.e., whether the two pathways have different outcomes both of which are required for dorsalization of the neural tube, or whether the dorsalization is induced by one of the signals while the other plays a permissive role. To clarify this issue, the direct effect of each pathway

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has been studied in this context. Wnt signals promote cell proliferation by up-regulating transcription of cyclin D1 (Burstyn-Cohen et al., 2004), whereas BMP signals are responsible for dorsal patterning (Liem et al., 1995, 1997; Chesnutt et al., 2004). BMP signals are also required for cell proliferation; however, this appears to be mediated by transcriptional up-regulation of Wnt1 (Burstyn-Cohen et al., 2004). Hence, BMP signals are mainly responsible for patterning, while the role of Wnt signals is to expand the populations of dorsal neuronal progenitors specified by BMP (Chesnutt et al., 2004), both of which are together required for making the dorsal part of the spinal cord.

It has, however, been noted in Wnt/β-catenin overexpression studies that the cell-proliferating effect of the Wnt/β-catenin pathway is best exerted in the ventral side of the neural tube, where BMP signals are absent (Cheung and Briscoe, 2003; Ille et al., 2007). In fact, cell proliferation promoted by activation of the Wnt/β-catenin pathway is counteracted by BMP signals in the dorsal neural tube. Similarly, neuronal differentiation caused by BMP signals is best achieved in the absence of Wnt signals. Hence, there is an underlying mechanism of mutual inhibition between Wnt and BMP pathways behind the scene of their cooperative function. Perhaps the negative feedback is taking place to maintain the balance of cell proliferation and differentiation.

Although the above studies in chick and mouse embryos suggest major roles for BMP signals in patterning, whereas for Wnt in proliferation, a study using zebrafish embryos clearly showed that Wnt signaling is required for both proliferation and patterning in the dorsal spinal cord (Bonner et al., 2008). In addition, it has been clarified that cell proliferation and patterning are independently regulated events; blocking cell proliferation does not affect dorsal–ventral patterning of the neural tube. This led authors to a further finding that these two events are regulated by different Tcf/Lef family members of the Wnt signaling pathway; Tcf3 mediates the Wnt/β-catenin signaling for proliferation, while Tcf7 (a.k.a. Tcf1) mediates the same signaling for dorsal neural tube patterning. This finding is significant in that Wnt signaling has a direct role in dorsal patterning, as does BMP signaling. In other words, the patterning process in the dorsal spinal cord is likely to involve a direct synergy of BMP and Wnt signaling rather than a secondary effect. These studies also highlight the situation where the same group of cells (dorsal spinal cord) integrate Wnt and BMP signals both synergistically and antagonistically depending on the task; synergistically for patterning and antagonistically for cell proliferation.

From the dorsal neural tube, neural crest cells delaminate, migrate, and differentiate into various cell types including peripheral neurons. While both Wnt and BMP signals support pluripotency of neural crest cells during the proliferation, these two signals are involved differently in the neurogenic differentiation process; while Wnt signals promote sensory neurogenesis, BMP signals suppress it (Kleber et al., 2005). This exemplifies not only that the roles of Wnt and BMP signals change during development, but also that the mode of crosstalk between the two signals changes as well.

Head Induction

Head and trunk induction has been a subject of intensive studies in the field of developmental biology as it is the basis for much of the vertebrate body plan (reviewed by Stern et al., 2006). In Amphibian embryos, Spemann's organizer provides inductive signals for axis formation. Spemann and Mangold have shown this by transplanting the dorsal lip of the blastopore (the organizer tissue) from a donor embryo to the ectopic (prospective ventral) region of a host embryo. This resulted in an embryo with a second dorsal body axis where the ventral side should have been. The secondary axis consisted of host cells except the notochord (which derives from the graft), proving the existence of inductive signals responsible for axis formation in the grafted tissue (Spemann and Mangold, 1924). It is now known from *Xenopus* studies that this secondary axis induction (with head and trunk structures) is recapitulated by inhibition of both BMP and Wnt signals (Glinka et al., 1997, 1998), while BMP inhibition alone often only induces a secondary axis without head structures (Suzuki et al., 1994; but see below). Spemann's organizer indeed expresses chordin and noggin, both of which function to inhibit BMP signals, and Dkk1, a Wnt inhibitor (reviewed by Niehrs, 2004).

In analogy to *Xenopus* embryos as mentioned above, failure to inhibit both Wnt and BMP signals in mouse also affects head formation: Head structures are not properly formed in double heterozygous for *Dkk1* and *noggin* (del Barco Barrantes et al., 2003). However, a similar phenotype is also obtained by a null deletion of *Dkk1* (Mukhopadhyay et al., 2001), or by double knock-out of *noggin* and *chordin* in which Dkk1 expression is not compromised (Bachiller et al., 2000). It is unclear whether in this context the Wnt and BMP pathways have distinct downstream targets, or, whether they function additively or synergistically to work on common targets.

Ventral Mesoderm Patterning in *Xenopus* Embryos

One of the first indications of instructive synergy between BMP and Wnt signals in vertebrates was shown in specification of the ventral mesoderm in *Xenopus* embryos (Hoppler and Moon, 1998). In *Xenopus* blastula, the equator region called marginal zone develops into mesoderm, which is further specified along the dorsal–ventral axis: The dorsal side of the marginal zone gives rise to the notochord and somites, whereas the ventral side develops into tissues such as pronephros kidneys and embryonic blood. Expressions of BMP4 and Wnt8 overlap in the ventral marginal zone, and indeed formation of ventral mesoderm requires both BMP and Wnt signals. Using the *vent* homeobox genes as molecular markers for ventral mesoderm, Hoppler and Moon showed the following: (1) *vent* gene expression requires both Wnt and BMP signals; loss of either (experimentally achieved with expression of dominant negative Wnt8 or dominant-negative BMP receptor Ia) results in reduced *vent* gene expression; (2) strong BMP signaling is

sufficient to induce vent genes; (3) Expression of *Wnt8* requires activation of the BMP pathway (i.e., dominant-negative BMP receptor expression results in a failure of *Wnt8* and *vent* gene expression). These results may be interpreted such as to suggest that BMP signaling functions upstream of *Wnt8* expression in a linear regulatory pathway. In other words, the function of BMP4 is to up-regulate Wnt8, which then induces *vent* expression. However, Wnt8 is not sufficient to induce *vent* gene expression (co-expression of dominant-negative BMP receptor and Wnt8 failed to induce *vent* expression). This means that activation of the BMP pathway is required for Wnt8 to exert its function to induce *vent* genes. It was later found that the *Xenopus vent2* promoter region contains BMP response elements where Smad proteins bind (Rastegar et al., 1999; Hata et al., 2000; von Bubnoff et al., 2005), but the mechanisms through which Wnt regulates *vent2* expression are still investigated. Very similar mechanisms have also since been discovered in other vertebrates (Ramel et al., 2005). This network of interactions between Wnt and BMP signaling in the ventral mesoderm would predict that strong inhibition of BMP signaling will not only cause an obvious lack of BMP signaling, but additionally, due to reduced Wnt8 expression, also a lack of Wnt signaling leading to complete dorsalization. Indeed, this is exactly what was observed when BMP signaling was completely blocked by injection of an inhibitory Smad (Tsuneizumi et al., 1997).

Stem Cells and Neural Induction

Embryonic stem (ES) cells undergo self-renewal proliferation while maintaining the potential to differentiate into a variety of cell types. Many studies have been conducted to search for factors that control differentiation of ES cells into desired lineages. Among those are studies to identify factors that cause neural differentiation (Gaulden and Reiter, 2008). Fibroblast growth factor (FGF) signaling and inhibition of BMP and Wnt signaling sum up the overall requirement for neural differentiation (Wilson et al., 2001; Kleber and Sommer, 2004; Bouhon et al., 2005). BMP and Wnt signals act on ES cells to maintain their pluripotency (Ying et al., 2003; Sato et al., 2004; Nusse, 2008). Thus, in the context of maintaining pluripotency and adopting neural cell fate, BMP and Wnt signals have similar effects. A question is raised as to whether these pathways have distinct functions or whether they are redundant. For the maintenance of pluripotency, either BMP or Wnt activation appears to be sufficient: A pharmacological Wnt signaling activator (GSK3β inhibitor) is sufficient to maintain the undifferentiated state of ES cells (Sato et al., 2004). Similarly, up-regulation of *Id* genes, direct downstream targets of the BMP pathway, is able to maintain self-renewal in the presence of LIF, without a need for serum (Ying et al., 2003). It is, however, unclear whether both of them are responsible for a common target or not.

With regard to neural induction in embryos, BMP inhibition was initially found to be sufficient for inducing the neural cell fate in *Xenopus* ectoderm; whereas BMP signals promote the epidermal fate (reviewed by Hemmati-Brivanlou and Melton, 1997). However, in the chick epiblast, BMP antagonism alone does not induce neural fate (Linker and Stern, 2004). This led to a search for additional factors and mechanisms required for chick neural induction (that had presumably been masked in *Xenopus* assays as they were endogenously supplied). One is FGF signaling, which initiates the neural fate in the medial epiblast (Streit et al., 2000; Wilson et al., 2000), and which was later also found to be required for neural induction in *Xenopus* (Delaune et al., 2005; Kuroda et al., 2005). FGF3 may function either in favor of inhibiting the BMP pathway or independently of BMP inhibition (Streit et al., 2000; Wilson et al., 2000, 2001; Pera et al., 2003). Another group of neural inducing factors identified turned out to encode Wnt inhibitors. Similar to BMP inhibition, Wnt inhibitors promote neural differentiation of epiblast cells that would otherwise adopt the epidermal fate (Wilson et al., 2001). This process requires endogenous FGF signaling, hence a model was proposed where Wnt signals in the lateral epiblast (future epidermal, non-neural ectoderm) inhibits cells to respond to FGF signaling by an unknown mechanism, which in turn allows BMP signals to promote epidermal ectoderm. In this context, the BMP pathway appears to be the key for the cell fate specification as seen in *Xenopus*: BMP inhibition (experimentally caused by a dominant-negative BMP receptor or by noggin) induces neural fate even in the presence of an FGF inhibitor (Wilson et al., 2000) or Wnt ligands (Wilson et al., 2001). Moreover, BMP sufficiently induces epidermal ectoderm even in the presence of a Wnt inhibitor (Wilson et al., 2001). However, high concentration of the FGF blocker or Wnt ligands inhibits the ability of noggin or dominant-negative BMP receptor to induce neural fate (Wilson et al., 2001). Thus, there remains a possible mechanism of BMP inhibition-independent neural induction, which might relate to regulation of FGF or Wnt signals.

Bone Formation

BMP was originally found as a factor promoting formation of cartilage and bone, hence its name bone morphogenetic protein (Wozney et al., 1988). It is interesting to note that, another protein similarly isolated based on its chondrogenic activity, turned out to encode an extracellular Wnt inhibitor (Hoang et al., 1996; originally named FrzB for Frizzled-related molecule expressed in Bone, now called sFRP3, for secreted Frizzled related protein 3). Since then, many studies have shown that the Wnt pathway is indeed involved in promoting bone formation (reviewed by Baron et al., 2006; Hartmann, 2006, 2007). This has been manifested by various mutations that primarily affect the Wnt pathway yet exhibit considerable phenotypes in bones. Although, contrary to the effect of sFRP3, most of studies suggest that activation of the Wnt pathway promotes bone formation. For example, loss-of-function mutation of a Wnt co-receptor LRP5 causes a low bone-mass phenotype (Gong et al., 2001), while its gain-of-function mutation causes hypermineralization of bones (Boyden et al., 2002; Little et al., 2002), known as a human syndrome high bone mass (HBM) trait, although it has recently been suggested that

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indirect mechanisms could contribute to this phenotype (Yadav et al., 2008). LRP6 single nucleotide polymorphism mutation impairing Wnt/β-catenin signaling results in low bone mass (Mani et al., 2007). Constitutive activation of β-catenin caused by APC (Adenomatous Polyposis Coli) deletion results in high bone deposition (Holmen et al., 2005). Moreover, Axin2 knock-out in mice causes skeletal defects, such as craniosynostosis where the skull fuses and ossificates at younger stages than normal (Yu et al., 2005; Liu et al., 2007). Finally, transgenic expression of stabilized β-catenin in osteoblasts causes high bone mass, accompanied by defects in osteoclast differentiation, as osteoprotegerin being as a target of Wnt/β-catenin pathway, while a loss of β-catenin in osteoblasts results in low bone mass (Glass et al., 2005; Holmen et al., 2005). All of these examples suggest that overactivation of the Wnt/β-catenin pathway promotes abnormal mineral deposits in bones while decreased Wnt/β-catenin signaling attenuates it, indicating that the pathway is responsible for regulating the right degree of bone formation and mineral deposition.

How is the Wnt/β-catenin pathway involved in bone formation? Why do opposite activities of the pathway (inhibition by sFRP3 and activation by β-catenin) both result in promotion of bone formation? What is the effect of Wnt pathway activation on the BMP pathway during bone formation? It appears that the interaction of the BMP and Wnt pathways is particularly complex in bone development, probably because the effect of the interaction differs depending on the developmental stage.

Most bones derive from mesenchymal precursor cells that have the ability to differentiate into osteoblast, adipocyte, or chondrogenic precursors, with an exception of the skull, which is formed by direct differentiation of neural crest-derived mesenchymal cells into bone tissues (reviewed in Baron et al., 2006; Hartmann, 2006, 2007). In skeletal bone formation, the fate of mesenchymal precursors is directed to osteoblast progenitors by activation of the Wnt/β-catenin pathway; without activation, mesenchymal precursors differentiate into chondrocytes or adipocytes (Day et al., 2005; Hill et al., 2005). Cells fated to become osteoblasts are, at this stage, called osteoprogenitors, in which the Wnt/β-catenin pathway functions to promote its proliferation and maintain the precursor status (i.e., attenuating further differentiation). BMP signals can stimulate those cells to become mature osteoblast (Amedee et al., 1994; Hughes et al., 1995). Hence, BMP and Wnt signals have opposing effects in osteoprogenitors. Once osteoprogenitors become osteoblasts, Wnt and BMP signals function cooperatively; both BMP2 and Wnt/β-catenin pathways promote further differentiation seen by expression of alkaline phosphatase (ALP; Bain et al., 2003; Rawadi et al., 2003) and mineralization (Holmen et al., 2005). Thus, the Wnt/β-catenin pathway is crucial at multiple steps of bone formation, and the interaction of Wnt and BMP signals is either opposing or cooperative depending on the differentiation step.

At the step during differentiation when mesenchymal precursor cells choose specific cell fates, Wnt and BMP signals have different roles. In mesenchymal cell line C3H10T1/2, which has the ability to differentiate into chondrocyte, adipocyte, muscle, or osteoblasts by extrinsic factors, BMP2 and β-catenin function distinctly. Muscle differentiation is promoted by β-catenin and not by BMP2 (Bain et al., 2003). On the other hand, chondrogenic differentiation is promoted strongly by BMP2 while β-catenin has no effect or rather functions inhibitory (Fischer et al., 2002; Bain et al., 2003). It was also seen in vivo that deletion of β-catenin in osteoblasts causes enhanced chondrogenesis and decreased osteogenesis (Day et al., 2005; Hill et al., 2005; Rodda and McMahon, 2006). Furthermore, in C3H10T1/2 cells, stabilized β-catenin strongly inhibits adipocyte differentiation while BMP2 does not affect it (Bain et al., 2003). It is interesting to note that, in the same cell line, exogenous BMP2 promotes TOPflash reporter activity (Bain et al., 2003). Hence, it appears that BMP2 functions in two ways; one to enhance the Wnt/β-catenin pathway and another to function independently of it, and these mechanisms are selectively used depending on the differentiation stage.

Despite the complex contribution of the two pathways, attempts have been made to pinpoint the function of each pathway at the final stage of osteoblast differentiation. In the induction of ALP expression, the ability of Wnt signals to up-regulate ALP is not blocked by cycloheximide, suggesting no requirement for new protein synthesis, while BMP2-dependent ALP induction is blocked, suggesting that the Wnt/β-catenin pathway plays a direct role in ALP induction (Rawadi et al., 2003). It was also shown in primary culture of mouse osteoblasts that defects in osteoblast differentiation caused by β-catenin deletion is not rescued by additional recombinant BMP2, although it normally increases osteogenic markers (Hill et al., 2005). In addition, sclerostin, a protein responsible for regulating the proper bone density, functions on Wnt signals, not BMP signals, for bone formation (van Bezooijen et al., 2004, 2007), despite its ability to bind BMP ligands (Kusu et al., 2003; Winkler et al., 2003). Furthermore, it was found in multiple myeloma patients that the myeloma cells secrete a soluble Wnt inhibitor, sFRP-2, which suppresses bone formation and causes bone destruction (Oshima et al., 2005). These studies suggest that the mineralization step in differentiated osteoblasts is much dependent on the Wnt pathway, consistent with the human conditions involving molecular lesions in genes encoding Wnt signaling components.

It has also been proposed in chondrogenesis that Wnt/β-catenin signaling plays a more instructive role than BMP signaling. Chondrogenic differentiation is characterized by Sox9-mediated transcriptional up-regulation of specific collagens (Lefebvre et al., 1997; Zhou et al., 1998; Bi et al., 1999; Akiyama et al., 2002). β-Catenin physically interacts with Sox9 and causes ubiquitination-mediated degradation (Akiyama et al., 2004; Jin et al., 2006). In this context, BMP2 blocks β-catenin–Sox9 interaction through activation of p38 MAPK. This suggests a mechanism where BMP signaling indirectly promote chondrogenesis by blocking Wnt/β-catenin signaling, which negatively works for chondrogenesis.

Tooth Development

Tooth development is a particularly rewarding area for studying the functional interaction of signaling pathways. Tooth formation involves tissue interactions between epithelium and underlying mesenchyme, mainly mediated by BMP, hedgehog (shh), and FGF signals (Tucker and Sharpe, 1999). Involvement of Wnt/β-catenin signaling, however, has also been suggested. For example, *Lef1−/−* mice lack both incisor and molar teeth along with lack of whiskers and hairs (van Genderen et al., 1994). *Axin2* mutant also displays tooth agenesis (Lammi et al., 2004). Overactivation of β-catenin promotes enlarged and ectopic tooth formation, which is accompanied by expansion of BMP4, Msx1/2, and Lef1 expression domains (Jarvinen et al., 2006; Liu et al., 2008). In contrast, overexpression of Dkk1 blocks teeth formation, which is accompanied by down-regulation of BMP and Msx1/2 expression domains (Liu et al., 2008). In the Dkk1-overexpressed tissues, the ability of BMP4 to induce *Msx1/2* is not affected, suggesting that Wnt/β-catenin signals are required upstream of BMP4 function (Liu et al., 2008). Another mouse mutant that exhibits a significant tooth phenotype is a knock-out of Wise (also called Ectodin/USAG-1/SOSTDC1; Kassai et al., 2005; Murashima-Suginami et al., 2007; Ohazama et al., 2008; Munne et al., 2009), a BMP inhibitor and also implicated as a Wnt modulator (Itasaki et al., 2003; Laurikkala et al., 2003; Yanagita et al., 2004). Targeted deletion of Wise/Ectodin shows supernumerary teeth, which is explained by an increase of either BMP or Wnt/β-catenin activity, based on the study of Liu et al (2008). A deletion mutant of LRP4, a negative Wnt signal regulator and also known as Megf7 (Johnson et al., 2005), shows the same phenotype as that of Wise/Ectodin mutant mice (Ohazama et al., 2008), suggesting that Wise/Ectodin may function on LRP4 in this context.

Limb Development

A role for Wise/Ectodin and LRP4 in crosstalk between Wnt and BMP signaling is also suggested in limb development (see below). Limb formation is a classic model for the study of morphogenesis. The process of limb development consists of induction and growth of limb buds, pattern formation along the three axes, and tissue differentiation (Tickle, 2006), which are regulated by multiple signaling cascades (Kengaku et al., 1998; Kawakami et al., 2001). In inducing the apical ectodermal ridge (marked by fgf8 expression), BMP signaling is required in the initial step and β-catenin functions subsequently to that; while at the later stage in dorsal–ventral patterning, β-catenin acts upstream of, or in parallel with, BMP signaling (Soshnikova et al., 2003). The role of Wnt and BMP signals further changes during the process of digit separation. Digits are formed in shape by programmed cell death of mesenchymal tissues at interdigital regions and the anterior and posterior margins of the limb buds. BMP ligands are expressed in the right place at the right time to induce cell death (Ganan et al., 1996; Yokouchi et al., 1996; Zou and Niswander, 1996). Indeed, when noggin is overexpressed by transgenesis in mouse, interdigital tissue is not completely regressed and thus extra-digits are formed, resulting in soft tissue syndactily (Guha et al., 2002; Plikus et al., 2004). However, despite the apparent reduction of BMP signals, the expression of downstream target genes, *Msx1* and *Msx2*, which are expressed in the interdigital regions and believed to be responsible for the cell death effect (Marazzi et al., 1997), are not affected in noggin-overexpressed limbs (Guha et al., 2002). Strikingly, Dkk1 is expressed in interdigits and its deletion mutant mice exhibit a soft tissue syndactily phenotype similar to the one seen in noggin transgenic mice (Mukhopadhyay et al., 2001). Because BMP2 induces Dkk1 expression as an immediate-early response, it is suggested that the apoptosis caused by BMP signals in normal limb development is mediated by Dkk1, rather than by expression of the direct BMP target *Msx1* (Mukhopadhyay et al., 2001; Guha et al., 2002). Deletion of a Wnt signal inhibitor LRP4 (also known as Megf7) also shows a similar phenotype of syndactily (Johnson et al., 2005). LRP4/Megf7 shows a strong homology to LRP5/6 at the extracellular domain while the intracellular domain shows little homology (for example, lacking "PPPSP" motifs that are required for signal transduction: Davidson et al., 2005; Zeng et al., 2005), hence predicted to work as a Wnt signal inhibitor by sequestering ligands.

Kidney Development

Reciprocal epithelial–mesenchymal interactions mediated by multiple signaling pathways are a fundamental aspect of vertebrate kidney development. In metanephros formation it involves two groups of tissues: Epithelial ureteric buds branch out of the Wolffian duct by signals derived from the metanephrogenic mesenchymal cells, which surround the ureteric buds and regulate further branching of the ureteric bud. In turn, at the tips of the branches, the ureteric epithelial cells induce mesenchymal condensation. The mesenchymal cells differentiate into different types of cells to eventually form nephrons, while ureteric bud gives rise to the collecting ducts and ureter. These processes require multiple signaling pathways including BMP and Wnt/β-catenin pathways (Schedl and Hastie, 2000; Perantoni, 2003; Carroll et al., 2005). BMP7 is enriched at the tip of the ureteric bud epithelium (Caruana et al., 2006), while BMP receptors are in both the branching epithelium and mesenchyme cells (Martinez et al., 2001). Mice with targeted deletion of BMP7 show severe dysgenesis of kidneys with little or no glomeruli, due to the failure of mesenchymal condensation at the initial stage (Dudley et al., 1995; Luo et al., 1995). Wnt4 and Wnt6 are expressed in ureteric buds and include tubulogenesis (Stark et al., 1994; Itaranta et al., 2002). Loss of β-catenin in the ureteric bud cell lineage causes defects in branches of ureteric epithelium, resulting in dysplasia or aplasia of kidneys (Bridgewater et al., 2008). Thus both Wnt and BMP pathways are required for kidney morphogenesis.

Because a very high dose of BMP7 signaling inhibits branching morphogenesis of ureteric buds (Piscione et al., 2001), an attempt was made to make a model animal of renal dysplasia by introducing a transgene expressing constitutively activated

ALK3 (BMP receptor Ia, the receptor for BMP2,4,7) in mouse. Rosenblum and colleagues then found that mice with high BMP signaling show elevated activity of the Wnt/β-catenin pathway revealed by Tcf-reporter transgene expression (Hu et al., 2003; Hu and Rosenblum, 2005). This led the authors to the discovery of a Smad1/Tcf4/β-catenin complex, which drives expression of *c-myc* in excess amounts (Hu and Rosenblum, 2005). Hence, both Wnt and BMP signals function synergistically in *c-myc* expression in the kidney.

In vitro analyses showed that BMP7 functions in a dose-dependent manner in kidney explants and in cell lines; low doses of BMP7 stimulate cell proliferation and tubular formation in a Smad1-independent manner, while high doses inhibit proliferation and induce apoptosis by means of activation of Smad1 (Piscione et al., 2001). The endogenous level of BMP7 plays beneficial roles for the recovery of renal cells from damages such as ischemia, injuries, and renal failure (Gould et al., 2002; Mitu et al., 2007). Both BMP7 and the BMP inhibitor Wise (also called USAG-1, SOSTDC1, and Ectodin) are abundantly expressed in adult kidneys (Yanagita et al., 2004), which may function to maintain the level of BMP7 signals beneficial for the kidney. After kidney damage, BMP7 plays a critical role in tissue repair (Wang et al., 2003; Zeisberg et al., 2003); while the BMP-antagonist Wise/USAG-1 prevents recovery; indeed, a mouse deletion mutant of Wise/USAG-1 shows a better than normal recovery from nephrotoxin-induced kidney damages, with prolonged survival of renal cells and preserved renal function (Yanagita, 2006; Yanagita et al., 2006). In addition, it was found in renal cell carcinoma that Wise/USAG-1 is down-regulated in 20 of 20 cases, although the mechanism is not known (Blish et al., 2008). Because Wise/USAG-1 has also been found to function as a Wnt signal inhibitor in different tissues (Itasaki et al., 2003) it is intriguing that other Wnt inhibitors such as sFRP1 and sFRP2 are also down-regulated in renal cell carcinomas (Gumz et al., 2007; Kawamoto et al., 2008).

Cancer

Involvement of deregulated activation of β-catenin in carcinogenesis is evident (Giles et al., 2003; Kikuchi, 2003; Logan and Nusse, 2004). While a wide variety of cancers show elevated β-catenin–dependent transcription, the causal relationship has been most clearly demonstrated in colorectal cancers. Approximately 80% of cases of colorectal cancer show mutations in APC, a protein required to degrade free β-catenin. In addition, 10% of cases show mutations in β-catenin itself, in the residues that normally get phosphorylated for degradation. Thus, 90% of colorectal cancers are associated with molecular lesions that cause overactivation of the Wnt/β-catenin pathway in the gut epithelium (Giles et al., 2003). It is noteworthy that down-regulation of the BMP pathway can also be a cause of intestinal cancer. Loss-of-function mutations of BMP receptor Ia (Howe et al., 2001; Zhou et al., 2001; He et al., 2004) or smad4 (Howe et al., 1998; Hohenstein et al., 2003) causes polyposis in colon. Overexpression of noggin by transgenesis in mouse also causes polyposis (Haramis et al., 2004; Batts et al., 2006). Furthermore, lacking one allele of smad4 increases the chance of developing malignancy in APC-deficient mice (Takaku et al., 1998). Thus BMP signals may antagonize β-catenin–dependent cell proliferation. In the normal gut, β-catenin dependent transcription is active at the bottom of crypts, where stem cells continue to proliferate (Sancho et al., 2003). In contrast, BMP4 is expressed in the intravillus mesenchyme and activates the BMP pathway in the overlying villi epithelium expressing BMPR1a (Haramis et al., 2004; Batts et al., 2006), while crypts express several BMP antagonists (Kosinski et al., 2007). Thus a balance is maintained between production of new cells in the crypt and differentiation of cells at the lumen/villi side: in the stem cell niche environment where the Wnt/β-catenin pathway is active at the bottom of the crypt, new epithelial cells are produced, which then move toward the lumen side where they cease proliferation and differentiate in response to BMP signals to renew the villi epithelium and function to absorb nutrients. This balance can be broken either when Wnt/β-catenin signaling is overactivated and cells continue to proliferate, or when BMP signal dependent differentiation is attenuated (Brabletz et al., 2009). It has been reported that colon cancer cells with stabilized β-catenin express significantly high levels of BMP4 (Kim et al., 2002), presumably reflecting a negative-feedback mechanism, which colon epithelial cells may have. It is also noted that colorectal cancer cell lines are resistant to BMP's tumor suppressing function (Nishanian et al., 2004).

Summary of Functional Interactions Between Wnt and BMP Signaling

Above examples of crosstalk between Wnt and BMP pathways reveal that the mode of interaction might be categorized into at least four groups (Fig. 3). The first is that these pathways have distinct roles at the same time, both of which contribute to a common goal or achievement (Fig. 3A). As seen in the dorsal neural tube, for example, Wnt and BMP signals are responsible for proliferation and patterning, respectively, both of which are required for formation of the dorsal neural tube. The second is that Wnt and BMP pathways seem to work on a common target, and two signals show additive or synergistic effects (Fig. 3B). This was clearly demonstrated in c-myc expression in the kidney. It is possible that they might play redundant roles in such contexts. The third is that Wnt and BMP signals function sequentially and have different roles in the course of developmental stages (Fig. 3C). As seen in osteogenesis and in the gut epithelium, Wnt signals promote proliferation and maintain undifferentiated status, while BMP signals cause differentiation. In those cases, while causing differentiation, BMP signaling blocks the effect of Wnt signals. This would prevent cells from receiving two signals that have opposing functions (maintaining undifferentiated status vs. causing differentiation), thus perhaps helping a smooth transition of differentiation processes. It is interesting to note that, once cells are differentiated, Wnt signals have different roles from the one at earlier stages, and cooperate with BMP signals during osteogenesis. The fourth case is that BMP

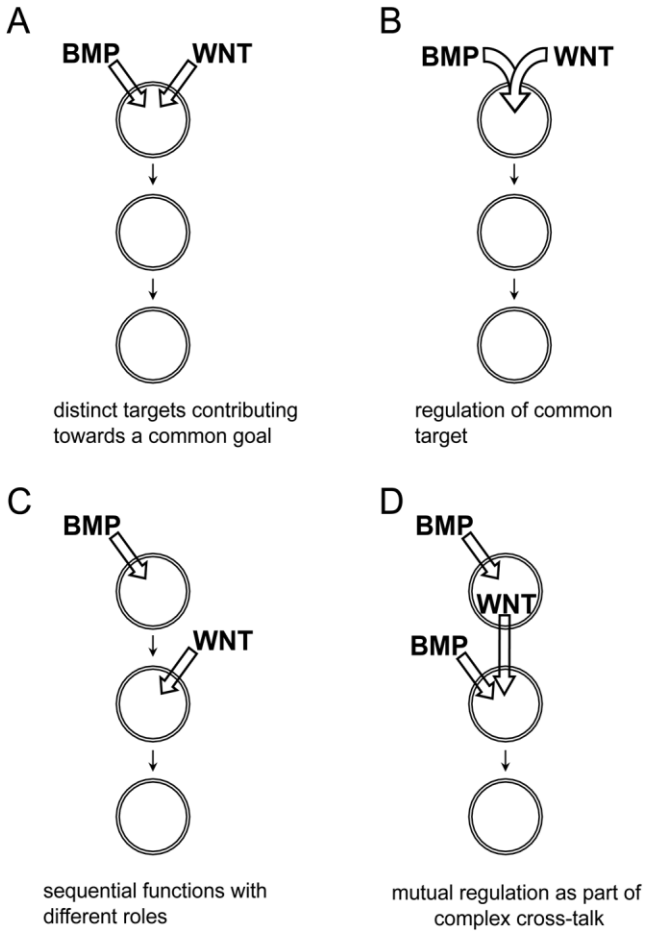

Fig. 3. Functional interactions between Wnt and bone morphogenetic protein (BMP) signaling. The four fundamental modes of functional interactions between Wnt and BMP signaling observed in a variety of tissues, illustrated on a highly generalized cell differentiation pathway. **A:** Wnt and BMP signaling independently regulate different targets in the same cells at the same stage, which are separately required and subsequently contribute toward a common biological goal. **B:** Wnt and BMP signaling integrate the regulation of a common target in the same cells, which leads to a biological outcome. **C:** Wnt and BMP signaling independently regulate distinct aspects of a cellular differentiation pathway at different stages of this differentiation pathway. **D:** Complex cross-regulation between Wnt and BMP signaling (in any of the above ways) additionally relies on regulation of expression of signaling components of one pathway by the other pathway. This generalized representation should, however, not distract from the tissue-, cell-, stage-, and sometimes gene-specific manners of interaction, which represent a fundamental aspect of integrated Wnt and BMP signaling.

signals induce expression of Wnt ligands, as seen in the dorsal neural tube and in the ventral mesoderm in *Xenopus* embryos (Fig. 3D). Once coexpressed, the two signals show further complex crosstalk. Up-regulation of Wnt ligand expression by BMP signaling suggests importance of having both signals in these contexts.

MOLECULAR INTERACTIONS BETWEEN BMP AND WNT SIGNALING

The prominent roles of combinatorial Wnt and BMP signaling in many different biological contexts explored in the first section raises the question about the molecular mechanisms by which Wnt and BMP signals can mediate this interaction. Exploration of these molecular mechanisms in this section generally reveals cell-type specific mechanisms that often cannot necessarily be extrapolated to other biological contexts.

Mutual Regulation of Gene Expression

In some cellular contexts, activation of the Wnt pathway leads to up- or down-regulation of BMP/transforming growth factor-beta (TGFβ) pathway components, or vice versa (Fig. 4A). For example, BMP4 is induced in colon cancer cells as a downstream target of β-catenin (Kim et al., 2002). In the developing limb mesenchyme, β-catenin up-regulates expression of BMP ligands and subsequent target *Msx* genes (Hill et al., 2006). In *Drosophila* leg development, Wnt and BMP repress each other's expression (e.g., Theisen et al., 1996). In other contexts, inhibitors of BMP signaling may be induced by Wnt/β-catenin (Xiro, Gomez-Skarmeta et al., 2001; BAMBI, Sekiya et al., 2004; PRDC, Im et al., 2007). Conversely, there are cases where BMP signals induce expression of Wnt and Frizzleds (Wnt3a, Fischer et al., 2002; Wnt1, Wnt3a, Rawadi et al., 2003; Fz6, Fz8, Yang et al., 2006). Expression of Lef/Tcfs can also be induced by BMP signals (Kratochwil et al., 1996; Dassule and McMahon, 1998; Nishanian et al., 2004), although in other contexts expression of these factors are inhibited by BMP2/4 (Jamora et al., 2003; Bonafede et al., 2006). These results again highlight the importance of cell type in determining the effect of cross-regulation.

Extracellular Regulation

There are several secreted molecules, which potentially bind to extracellular components of both the BMP and Wnt pathways, thus affecting both signals (Fig. 4B). Cerberus, a secreted molecule isolated as a head inducer in *Xenopus* (Bouwmeester et al., 1996), has been shown to bind BMP, Nodal, and Wnt ligands and to inhibit these signals. By doing so, Cerberus has the ability to promote head formation (Silva et al., 2003). Other secreted molecules include CTGF (connective tissue growth factor, newly named as CCN2), which binds BMP4, TGFβ1 (Abreu et al., 2002), and LRP6 (Mercurio et al., 2004); Wise (USAG/Ectodin/SOSTDC1), which binds BMPs (Laurikkala et al., 2003; Yanagita et al., 2004) and LRP6 (Itasaki et al., 2003); and Sclerostin, which binds BMPs (Kusu et al., 2003; Winkler et al., 2003) and LRP5/6 (Li et al., 2005; Semenov et al., 2005; Ellies et al., 2006). As already mentioned in the

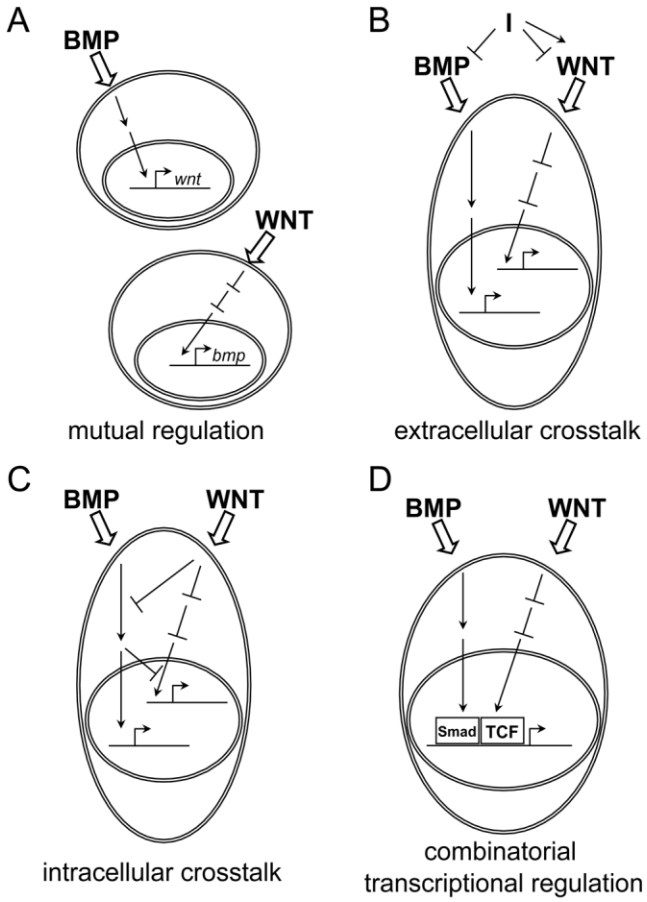

Fig. 4. Molecular interactions between Wnt and bone morphogenetic protein (BMP) signaling. Wnt and BMP signaling mechanisms tend to interact in four fundamentally different ways. **A:** By mutual regulation of each other's gene expression (i.e., BMP regulates Wnt and Wnt regulates BMP gene expression), either positively (as illustrated) or negatively. **B:** By extracellular molecules targeting ligands or receptors of both pathways, causing either activation or inhibition of signaling. **C:** By interactions between signal transduction components of the pathways, causing interference with or enhancement of one pathway by signal transduction components of the other pathway. **D:** By integrating the signal transduction mechanisms of both pathways in a synergistic or antagonistic way by means of *cis*-regulatory enhancer and promoter sequences to regulate target gene expression.

previous section on bone formation, a loss-of-function mutation of the sclerostin-encoding gene, *Sost*, is responsible for sclerosteosis, characterized by increased bone density (Kusu et al., 2003; Winkler et al., 2003; van Bezooijen et al., 2004). Sclerostin can inhibit bone formation either by blocking BMP signaling through its interaction with BMP ligands, and/or, by binding to LRP6 and thus by interfering with Wnt signaling, of which the latter appears to be the likely primary effect (van Bezooijen et al., 2004, 2007; ten Dijke et al., 2008). Other secreted molecules can also play roles in connecting BMP and Wnt signals. For example, one of the soluble Frizzled-related proteins, Sizzled, was identified as a Wnt antagonist (Salic et al., 1997) and turned out to function as an inhibitor of Xlr (*Xenopus* Tolloid-related), a metalloprotease which degrades the BMP inhibitor chordin (Lee et al., 2006). Thus generally extracellular mechanisms regulating both Wnt and BMP signaling mediate simultaneous repression of both signaling pathways.

Intracellular Regulation

Some signal transduction components of the Wnt and BMP pathways are found to interact with each other (Fig. 4C). One mechanism accounting for antagonism between Wnt and BMP at the cytoplasmic level is through a direct interaction of Dishevelled and phosphorylated Smad1, as was demonstrated in bone marrow stromal cells (Liu et al., 2006). As mentioned earlier, Wnt signals function to promote proliferation of mesenchymal stem cells and to assist in maintaining the undifferentiated status, whereas BMP signals stimulate differentiation, thus highlighting opposing functions of these two signals. Moreover, in bone marrow stromal cells, activation of the Wnt pathway by Wnt3a is antagonized by BMP2. A possible mechanism for this antagonism was presented in vitro (Liu et al., 2006). In the absence of exogenous Wnt and BMP ligands, Dishevelled-1 is bound to Smad1 at its linker region, which is located between two Mad homology domains MH1 and MH2. Stimulation of cells with Wnt3a results in dissociation of the Dishevelled-Smad1 formation, which allows transduction of Wnt signals. Intriguingly, when cells are stimulated with both Wnt3a and BMP2, Smad1 is phosphorylated and the interaction between Dishevelled-1 and Smad1 is further enhanced, thus Wnt3a-dependent stabilization of β-catenin is attenuated. When Smad1 is mutated such that it cannot be phosphorylated by BMP signaling, Wnt pathway activation is not antagonized by BMP2 (Liu et al., 2006). This is a possible mechanism whereby BMP signals inhibit the Wnt pathway. However, it is so far unclear whether similar mechanisms operate in other cells and other biological contexts.

Another cell type, mouse embryonic maxillary mesenchymal cells, was also used to demonstrate the formation of a complex of Dishevelled-1 and Smad3. However, in this case, TGFβ enhances the activity of the Wnt-responsive reporter TOPflash and Smad3 binds to Dishevelled-1 through the MH2 domain (Warner et al., 2005a,b). It is uncertain what accounts for the opposite outcomes; BMP versus TGFβ signaling (Smad1 or Smad3), or cell type-specific mechanisms.

Recently, mechanisms emerged linking GSK3 function with the BMP pathway, which involves Smad1 phosphorylation by GSK3 (Fuentealba et al., 2007). GSK3 phosphorylates Smad1 at specific sites in the linker region that causes ubiquitination and subsequent degradation of Smad1. The ability of GSK3 to phosphorylate Smad1 is de-

pendent on its prime-phosphorylation at the nearby sites by MAP kinase (MAPK). Earlier work had indeed demonstrated that FGF-MAPK signaling inhibits the BMP pathway through Smad1 phosphorylation at the linker region (Pera et al., 2003; Sapkota et al., 2007). When GSK3 is inhibited, Smad1 activation by BMPR (phosphorylation of Smad1 at the MH2 domain by BMPR1) is maintained at least a few hours longer compared with the situation where GSK3 is active (Fuentealba et al., 2007). Thus the duration of BMP signaling is regulated by GSK3 activity.

Direct interaction of β-catenin with inhibitory Smad molecules has been shown as another mode of crosstalk between Wnt and BMP/TGFβ signaling. In skin epidermal cells, overexpression of Smad7 causes inhibition of the Wnt/β-catenin pathway, which was explained by direct binding of smad7 to β-catenin along with a E3 ligase Smurf2, causing degradation of β-catenin (Han et al., 2006). The physical interaction of Smad7 and β-catenin was also seen in COS1 cells, where the binding is enhanced by TGFβ signaling (Edlund et al., 2005). In this case, however, complex formation of Smad7 with β-catenin and Tcf/Lef1 does not result in degradation of β-catenin; but rather enhances Wnt pathway activity by further promoting formation of the β-catenin/LEF1 transcription complex. Indeed, Smad7 promotes TGFβ-induced association of β-catenin and LEF1. Hence, interaction between β-catenin and smad7 can have opposite outcomes, depending on the cell type. Furthermore, in another in vitro context, Smad7 stabilizes β-catenin by binding to Axin and thus dissociating β-catenin from the degradation complex (Tang et al., 2008). Although, in this case β-catenin proteins stabilized by Smad7 associate with the cadherin complex and promote cell–cell adhesion.

Mapping the active areas of the Wnt and BMP pathways in *Xenopus* blastula and gastrula has provided a unique depiction of the spatiotemporal regulation of both pathways (Schohl and Fagotto, 2002). It was revealed that, while the regional activities of each pathway do not respect tissue boundaries, the active areas of each pathway strikingly overlap, suggesting a possible cross-regulation between the pathways. Further experimental studies revealed that the phosphorylation status of Smad1 and Smad2 is dependent on β-catenin (Schohl and Fagotto, 2002), suggesting a regulatory effect of the Wnt pathway on BMP and TGFβ signals. This is also in agreement with an earlier report describing reciprocal enhancement of Wnt/β-catenin and TGFβ/Smad2 pathways in the organizer region (Crease et al., 1998).

Another possible level of interaction between Wnt and BMP signals in the cytoplasm involves the PI3k/PKB pathway. PI3 kinase, which resides in the cytoplasm and is recruited to growth factor receptor tyrosine kinases upon their stimulation, phosphorylates PIP2, thereby converting it to PIP3. PIP3 in turn recruits and activates protein kinase B (PKB, also known as Akt), which leads to the promotion of cell survival and to blocking of apoptosis (Nicholson and Anderson, 2002). PTEN, a phosphatase and a major tumor suppressor gene, converts PIP3 back to PIP2 (Cantley and Neel, 1999; Maehama and Dixon, 1999; Mutter, 2001). A role of PTEN in Wnt/β-catenin signaling was shown in the PTEN-null prostate cancer cell line, PC3, which displays significant accumulation of β-catenin in the nucleus (Persad et al., 2001). Re-introducing PTEN in PC3 cells reduces the amount of β-catenin and represses TOPflash reporter activity (Persad et al., 2001). Activated PKB has a wide array of functions, one of which is to phosphorylate GSK3β and thereby to inhibit its activity. Hence, one can explain the function of PKB as inactivating GSK3β thus resulting in stabilizing β-catenin (Haq et al., 2003). Intriguingly, in the study of intestinal epithelial cells, He et al. showed that the balance of BMP and Wnt signaling activities (hence, the balance of differentiation and stem-cell maintenance) is mediated by PTEN/PKB signaling. In the presence of excess BMP4, the majority of PTEN is in the active form (nonphosphorylated) while excess noggin increases the phosphorylated form of PTEN. Thus, in differentiated intestinal epithelium, the active form of PTEN attenuates the PI3 kinase activity, resulting in high GSK3β activity and repressed Wnt/β-catenin pathway (He et al., 2004).

The effect of PKB-phosphorylated GSK3β on β-catenin is, however, uncertain. Kinase activity of GSK3β is dependent on the presence of a prime-phosphorylation on the target substrate. PKB phosphorylates GSK3β at position Ser-9. Residue Arg-96 of GSK3β, which interacts with the prime-phosphate of the substrate, can also interact with its own phosphorylation on Ser-9 (Dajani et al., 2001; Frame et al., 2001), providing thus a mechanism for negative regulation based on competition between the internal pseudosubstrate and a real target. Although this mechanism applies to the case of PKB inhibiting GSK3β's kinase activity toward glycogen synthase (Frame et al., 2001), the case for PKB-mediated inhibition of GSK3β as a mechanism for activating β-catenin signaling is more ambiguous (Ding et al., 2000). Phosphorylation of GSK3β at Ser-9 by insulin-induced PKB is not sufficient to stabilize β-catenin (Ding et al., 2000). Moreover, Ser-9 is dispensable for the inhibitory effect of GSK3β on the Wnt/β-catenin pathway in HEK293 cells (Ding et al., 2000). In addition, phosphorylation of β-catenin by GSK3β can occur in the absence of Arg-96 (Frame et al., 2001). Hence, PKB-mediated phosphorylation of GSK3β may not necessarily be inhibitory on β-catenin and the mode of action of GSK3β can be different depending on the substrate.

At the level of translocation of β-catenin to the nucleus, a possibility of enhancing the Wnt pathway by TGFβ signals was proposed. TGFβ1 can promote nuclear accumulation of β-catenin in a Smad3-dependent manner (Jian et al., 2006). Clarifying the actual mechanism requires a better understanding of how nucleocytoplasmic shuttling of β-catenin is regulated.

Regulation at the Promoter or Enhancer Level

Perhaps the most compelling cases of synergy between BMP and Wnt signals are those where target gene expression is directly regulated by these signals at the promoter level (Fig. 4D). Upon Wnt signaling activation, β-catenin translocates into the nucleus and binds the Lef/Tcf family members of transcription fac-

tors, where it functions as a transcriptional co-activator. Tcf/Lefs (Tcf1, Tcf3, Tcf4, and Lef1) contain a HMG DNA-binding domain, through which they bind well-conserved DNA sequences (Korinek et al., 1997; Barker et al., 2000; Hoppler and Kavanagh, 2007). The DNA sequence mediating Smad4 binding in response to BMP signals has also been identified (Hata et al., 2000; Korchynskyi and ten Dijke, 2002; von Bubnoff et al., 2005). Many genes have been identified that harbor both Smad and Tcf/Lef binding sites within their regulatory sequences. These include *Tbx6* (Szeto and Kimelman, 2004), *Msx2* (Willert et al., 2002; Hussein et al., 2003), *Xtwin* (TGFβ, not BMP; Labbe et al., 2000; Nishita et al., 2000; Letamendia et al., 2001), *Emx2* (Theil et al., 2002), *Slug* (Sakai et al., 2005), *c-myc* (Hu and Rosenblum, 2005); and *Ultrabithorax* (*Ubx*; Fig. 2B; Saller et al., 2002), *even-skipped* (*eve*; Fig. 1; Lee and Frasch, 2005), and *distal-less* (*dll*) in *Drosophila* (Estella et al., 2008). In most cases, the expression is synergistically enhanced by the simultaneous activation of both BMP and Wnt pathways compared to each alone. Transcriptional regulation of *Msx2* has been intensively studied in view of the synergistic effect of Tcf/Lef and Smad binding elements (Hussein et al., 2003). When Smad binding sites are removed from the regulatory region, β-catenin does not fully activate transcription even if Lef/Tcf binding sites are left intact. Similarly, BMP signals or Smad4 proteins do not induce full transcriptional activity when Tcf/Lef binding sites are abolished. Analogous results were also found in the synergy of Wnt and activin/nodal or TGFβ signaling on *Xtwin* and *gastrin* expression (Nishita et al., 2000; Lei et al., 2004). These results were attributed to the complex formation of β-catenin, Tcf/Lef, and Smad proteins that apparently facilitate the transcriptional activity of these proteins on the promoter (Labbe et al., 2000; Nishita et al., 2000; Letamendia et al., 2001). Studies using chromatin immunoprecipitation or DNA affinity precipitation methods have revealed that Lef/Tcf proteins are found in the complex of Smad response elements and Smad proteins (Hussein et al., 2003; Lei et al., 2004). Moreover, in regulation of *Msx2* expression, Lef1 facilitates BMP2 signal-dependent transcriptional activation irrespective of its β-catenin binding (Hussein et al., 2003). Similarly, Smad4 is found in the complex of β-catenin and Tcf/Lef, which is bound to the Tcf/Lef response element of DNA (Hussein et al., 2003; Lei et al., 2004). Smad4 forms a complex with β-catenin and Tcf/Lef even in the absence of BMP2 signals, suggesting that the endogenous level of Smad4 (or activated Smad4 by the endogenous level of BMP signals) contributes to the β-catenin–dependent transcription, which can be further promoted by additional BMP2 signals (Hussein et al., 2003). The complex of Lef/Tcf and Smad proteins may be formed not only with Smad4 but also with Smad1 (Theil et al., 2002) or Smad3 (Labbe et al., 2000; Lei et al., 2004), all of which have been shown to promote expression of target genes. Hence, interaction of Smad proteins and Tcf/Lef plays a critical role in regulating transcriptional activity of target genes that have promoters or enhancers containing binding sites for both.

Contrary to these synergistic effects, antagonistic effects of Smad proteins on the Tcf/Lef and β-catenin complex have also been shown at the promoter or the enhancer level. *Drosophila* genetics has recently uncovered mechanisms through which BMP interferes with Wnt signaling in the tissues that later develop into the wing of the adult fly (Zeng et al., 2008). BMP signaling-activated Smad1/5/8 (Mad) can bind to Tcf (pangolin) and interfere with β-catenin (Armadillo) binding by competition, thus preventing expression of Wnt target genes such as the homeobox gene *distal-less*. This antagonistic effect is in contrast to the effect of the Smad–Tcf complex seen in vertebrates as above, which mediates synergy between Wnt and BMP signaling. Although, organism-specific mechanisms could account for this dramatic difference in outcome. It is interesting to note that inhibition of Wnt signaling in the developing *Drosophila* wing tissue happens irrespective of the particular target gene, as it is even evident in Topflash reporter experiments (Zeng et al., 2008).

PERSPECTIVES

BMP and Wnt signaling pathways are essentially independent signaling pathways, which are able to function individually. However, they tend to regulate similar biological processes in the same tissues and cells, leading to functional interactions. As we reviewed in the first part, there are essentially four different modes in which these functional interactions between Wnt and BMP signaling happen at the tissue and cellular level (Fig. 3). In the second part, we reviewed the various molecular mechanisms that have evolved to mediate these functional interactions and integrate BMP and Wnt signaling at the level of the protein components of these signaling pathways (Fig. 4). One of the purposes of this review is to highlight and emphasize the fact that cross-interactions of BMP and Wnt signaling may occur at various levels in the cascade, and that the consequence/outcome can vary depending on cell types, developmental stage, or even the particular target gene. This complexity of interactions between BMP and Wnt signaling should not be perceived as a distracting complication, but should be recognized as a necessary and powerful mechanism for conferring diverse functions to various tissues and for creating the magnificently sophisticated structure of our bodies.

While the crosstalk between BMP and Wnt signaling is perhaps the most prominent and obvious example for combinatorial signaling in embryonic development and regulation of stem cells, interaction between Wnt signaling and other signaling pathways is perhaps a general feature of the role of Wnt signaling in biology. As suggested by Martinez-Arias and colleagues, the role of Wnt signaling in some contexts may be, at least in part, to stabilize transcriptional events caused by other mechanisms and to eliminate unwanted transcriptional activities ("filtering noise"), rather than playing an instructive role by itself (Arias and Hayward, 2006). This suggests that regulators of the Wnt pathway (including the pathway components

themselves) carefully monitor cellular activities and functions as a watchdog. In turn, the Wnt pathway may be readily influenced by the biological events taking place in the cell, and hence the pathway activity is susceptible to that of other signal transduction pathways. The challenge for future research will be to discover these combinatorial mechanisms to understand the full contribution of Wnt signaling to regulation of cell behavior and differentiation. Combinatorial signaling mechanisms will therefore prove to be at least as important for developmental biology as the well-studied linear signal transduction pathways, even if—or maybe exactly because—they are cell type-specific.

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