BMP signaling is required for the generation of primordial germ cells in an insect

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Accessibility
Two modes of germ cell formation are known in animals. Specification through maternally inherited germ plasm occurs in many well-characterized model organisms, but most animals lack germ plasm by morphological and functional criteria. The only known alternative mechanism is induction, experimentally described only in mice, which specify germ cells through bone morphogenetic protein (BMP) signal-mediated induction of a subpopulation of mesodermal cells. Until this report, no experimental evidence of an inductive germ cell signal for specification has been available outside of vertebrates. Here we provide functional genetic experimental evidence consistent with a role for BMP signaling in germ cell formation in a basally branching insect. We show that primordial germ cells of the cricket *Gryllus bimaculatus* transduce BMP signals and require BMP pathway activity for their formation. Moreover, increased BMP activity leads to ectopic and supernumerary germ cells. Given the commonality of BMP signaling in mouse and cricket germ cell induction, we suggest that BMP-based germ cell formation may be a shared ancestral mechanism in animals.

**Significance**

Many model organisms specify germ cells using maternally supplied germ-line determinants. In contrast, mice rely on embryonic cell–cell signaling to induce cells to become germ cells. Molecular evidence for inductive germ-line specification had previously been provided only for the mouse. Here we provide functional evidence for inductive germ cell specification in an invertebrate, by showing that bone morphogenetic protein (BMP) signaling, which induces mouse germ cell specification, is required for establishment of embryonic germ cells in a cricket. BMP pathway knockdown causes reduction or loss of germ cells, and elevated levels of BMP signaling cause supernumerary and ectopic germ cells. BMP-based germ cell induction in mice and crickets suggests that this may be a shared ancestral mechanism in animals.

*Gryllus* PGCs first arise among the abdominal mesoderm 2.5 d after egg laying (AEL) (4). To determine whether *Gryllus* PGCs actively transduce BMP signals during their formation, we used multiplex immunostaining to simultaneously detect the PGC marker *G. bimaculatus* *piwi* orthologue (Gb-Piwi) (4) and the BMP signal effector phosphorylated Mad (pMad) (20). We observed coexpression of high levels of Gb-Piwi and nuclear pMad in mesodermal cells of anterior abdominal segments, revealing that there is active BMP signaling in PGCs at the time that they are specified, and in most PGCs while they coalesce into clusters (Fig. 1 A–C”). We also detected nuclear pMad in both ectodermal and mesodermal cells. This somatic expression was detected at the highest levels laterally (dorsally) and decreased toward the medial (ventral) region, becoming undetectable at 7–10 cell diameters from the lateral (dorsal) edge of the embryo along the entire anterior–posterior axis (Fig. 1 A–C and SI Appendix, Fig. S2 A–F”). This graded expression pattern is consistent with a conserved role for BMP signaling in dorsoventral patterning (21). The expression of nuclear pMad in PGCs during their formation is also consistent with a role for BMP signaling in PGC specification.

Next, we examined the expression of multiple BMP pathway members in *Gryllus* embryos during PGC formation. *Gryllus* orthologs of vertebrate ligands BMP2/4 (*G. bimaculatus* decapentaplegic, Gb-dpp1 and Gb-dpp2) and BMP5/7/8 (*G. bimaculatus* glass bottom boat, Gb-gbb) are expressed in the abdomen throughout the period of PGC formation, and are enriched at the dorsolateral margins of the embryo by 4 d AEL (Fig. 1 D–F” and SI Appendix, Figs. S1 and S2 D–F”). Double labeling of BMP ligand expression and Gb-Piwi (4) confirmed that the BMP ligands Gb-dpp1 and Gb-dpp2 are expressed within 2–4 cell diameters of the PGCs (Fig. 1 D–F”), and Gb-gbb is expressed in cells adjacent to the PGCs (Fig. 1 F”). BMP receptors *Gryllus* *bimaculatus* thickveins

**Results and Discussion**

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BMP signaling is required for the generation of primordial germ cells in an insect

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(Gb-tnv) (type I) and Gryllus bimaculatus punt (Gb-pnt) (type II) and the BMP effector Smad1/5/8 (Gryllus bimaculatus Mothers against dpp, Gb-Mad) are expressed ubiquitously, including in PGCs, throughout PGC specification and cluster coalescence. In stage 7 embryos, Gb-dpp1 is expressed in ectodermal foci in each segment, and Gb-dpp2 and Gb-gbb are expressed along the dorsal edge of the embryo. Abdominal segments A1–A3 are shown; arrowheads indicate strong ligand expression. (D–F) Embryos double-stained for BMP ligands (green, indicated by arrowheads) and Piwi protein (magenta, single optical section, indicated by arrows). (G–J) Receptors Gb-tkv and Gb-put are expressed ubiquitously throughout embryogenesis, including in PGCs. (G, G′, J′, and J) A dorsal focal plane of stage 5 embryos, where PGCs form. White outlines in H, H′, J, and J′ indicate PGC clusters in stage 7 embryos. Anterior is up; A2 is the anteriormost segment shown in each panel. [Scale bar, 10 μm in A′′′, B′′′, C′′′, G, and H (also applies to G′–J′), and 50 μm in all other panels.]
However, the complete absence of PGCs in a significant proportion of Gb-Mad and Gb-gbb RNAi embryos and the absence of apoptotic PGCs in all RNAi experiments suggest a direct requirement for BMP signaling in Gyrillus PGC specification.

We hypothesized that if BMP signaling acts as a direct inductive signal in PGC specification, then elevated or ectopic levels of BMP signaling might increase the number of PGCs formed or cause them to form in ectopic locations. To test this hypothesis, we took two approaches to raise BMP signaling activity in early embryos above wild-type levels: (i) injecting 4′-hydroxychalcone, a recently identified chemical activator of the BMP pathway (30), and (ii) injecting recombinant D. melanogaster Decapentaplegic (Dpp) protein (Dm-Dpp) (SI Appendix, Figs. S8 and S9). In both treatments, axial patterning, mesoderm formation, and overall embryonic morphology appeared normal in experimental embryos, but nuclear pMad levels at the time of PGC specification were slightly elevated and expanded toward the ventral midline with respect to controls (Fig. 3 M–O and SI Appendix, Fig. S10 A–D). PGC cluster size showed a dose-dependent increase in every segment of treated embryos (Fig. 3 D–F’ and SI Appendix, Fig. S10E). Summing the volumes of all PGC clusters in an embryo showed that the total number of PGCs per embryo was increased by up to 42% (Fig. 3G), consistent with activation of BMP signaling. In addition, both treatments caused a significantly increased frequency of ectopic PGCs, defined as PGCs found in segments A1, A5, or A6 (10 mM 4′-hydroxychalcone, P < 0.01, n = 31, Fig. 3 E’ and L; 10 μg/mL Dm-Dpp, P < 0.01, n = 23, Fig. 3 F and J–L; 100 μg/mL Dm-Dpp, P < 0.05, n = 15, Fig. 3 F’ and L). Ectopic and supernumerary PGCs were not randomly distributed, but instead were clustered adjacent to the coelomic pouches like wild-type PGCs (Fig. 3 C, C’, I, and K). Ectopic PGCs are unlikely to result from mismeigration, as Gyrillus PGCs arise in the segments that will house the gonad primordium (A2–A4) and, unlike PGCs in Drosophila or mice, do not undergo long-range migration (4). To definitively eliminate the possibility of BMP-induced mismeigration, one would need to track PGCs in vivo, a technique that is not currently possible in Gyrillus.

Some embryos treated with Dm-Dpp displayed an increase in the proportion of A2–A4 segments lacking PGCs (SI Appendix, Fig. S10 F and H). We note, however, that a low level of PGC loss in these segments can be a nonspecific consequence of embryo perturbation, even in buffer-only injected controls, and that the proportion of these segments lacking PGCs in 4′-hydroxychalcone-treated embryos was not significantly different from controls (SI Appendix, Fig. S10 F–H). We think it unlikely that BMP signaling actively represses PGC formation, as this hypothesis does not explain the supernumerary or ectopic PGCs induced by the BMP activation treatments. However, the altered distribution of PGCs induced by widespread BMP activation suggests that PGC specification is not solely dependent on BMP levels, but instead may involve a range of acceptable BMP signal levels integrated with
additional segment-specific positional information. This hypothesis is additionally consistent with the expression of nuclear pMad during PGC formation (Fig. 1A–C and SI Appendix, Fig. S2A–A”), as the presence of nuclear pMad in some non-PGC cell types suggests that additional mechanisms may be involved in PGC specification. In mice, where BMP signaling induces PGC formation,
phosphorylated SMAD is also detectable in multiple somatic cells, including cells neighboring the nascent PGCs (31). A combination of antagonistic and competence signals operate together with BMP signaling to ensure mouse PGC specification in only a subset of phosphorylated SMAD-positive cells, and we hypothesize that analogous mechanisms may be operative in Gryllus. Thus, although we cannot formally rule out additional roles for BMP signaling in migration, maintenance, and/or proliferation of PGCs, we propose that our results are most consistent with the hypothesis that BMP signaling induces PGC specification in Gryllus.

We have provided evidence that cricket PGCs are specified via BMP signaling, which appears to be necessary for cricket PGC formation. These data represent, to our knowledge, the first functional genetic evidence for an inductive mode of invertebrate germ cell specification. Our results support a model whereby Gb-dpp signals (and to a lesser extent Gb-dpp1 signals) from the dorsolateral abdominal ectoderm are transduced via Gb-Mad in the adjacent mesoderm and cause some of those cells to adopt PGC fate. The presence of supernumerary germ cells in BMP activation experiments and the reduction in PGC cluster size in RNAi experiments raise the possibility that BMP signaling promotes PGC proliferation as well as, or rather than, specification. However, this hypothesis does not account for the presence of ectopic PGCs, given that Gryllus PGCs do not undertake long-term migration (4), nor for the complete absence of PGCs in Gb-gbh and Gb-Mad RNAi experiments, given that PGC clusters are derived from a large number of initially specified precursors rather than just one or a few (4).

The fact that ectopic germ cells are found only in lateral positions may be because although pMad levels are elevated by both treatments (Fig. 3 M–O), the shape of the activation profile is the same as that of wild-type embryos: nuclear pMad is at the highest levels dorsolaterally, where PGCs are specified, and at the lowest levels ventrally, where PGCs are never observed (SI Appendix, Fig. S10 B and D). We hypothesize that an inhibitory ventral signal, possibly of the sog/Chordin family, may restrict BMP signaling to dorsal tissues. Alternatively or in addition, a lateral competence signal may play a role in ensuring that not all cells expressing high levels of nuclear pMad differentiate as PGCs. With respect to their specification at the correct position along the anterior–posterior axis, the mechanism(s) that restricts PGC formation to the lateral mesoderm of segments A2–A4 in wild-type embryos are currently unknown. The appearance of ectopic PGCs anterior and posterior to wild-type positions in BMP activation experiments suggests that sufficiently high BMP signaling activity may perturb or overcome the positional information, potentially Hox gene-mediated, that normally limits PGC specification along the anterior–posterior axis. Our observation of segment-specific effects on PGC number in both the RNAi and BMP activation experiments is consistent with this model, as it suggests that PGC sensitivity to BMP signaling varies along the anterior–posterior axis.

Our findings in the cricket are similar to those observed in mouse PGC specification. In both cases, an ectodermal BMP signal induces a competent subset of mesoderm to become PGCs (32). PGC phenotypes are observable only in mildly knocked down Gryllus embryos, or in heterozygous knockout mice (8–10), as strong RNAi phenotypes and homozygous knockout conditions severely disrupt early development. Loss of BMP2/4, BMP8, and SMAD1/5/8 orthologs leads to loss or reduction of PGCs in both systems (Fig. 4 A). Two parsimonious evolutionary scenarios based on functional genetic data that could explain the mechanistic similarities observed in cricket and mouse PGC specification are that BMP-based PGC induction (i) originated in a Bilaterian ancestor (denoted by a red diamond in Fig. 4B) or (ii) evolved independently within annelids and insects (red text in Fig. 4B). Given that, to our knowledge, a role for BMP signaling in PGC formation has been directly tested in only two phyla, at present it is difficult to determine which of these hypotheses is more likely. However, in support of the first hypothesis, we note that in multiple metazoan phyla, germ cells are responsive to BMP signaling at some stage of their development, including PGC specification, gametogenesis, germ-line cell divisions, and adoption of germ cell fate by stem cells in culture (Fig. 4B and SI Appendix). This suggests that in addition to its conserved role in dorsoventral patterning (21), there is an ancient and widespread association of BMP signaling with germ cell fate or pluripotency. If future studies reveal a role for BMP signaling in PGC formation in additional taxa that use the inductive mode, that

**Fig. 4.** BMP signaling in germ cells across the Metazoa. (A) Hypothetical model for BMP pathway function in cricket and mouse PGCs. Homologous proteins are shown in the same color. Black outlines indicate molecules whose role in germ cell formation has been tested experimentally through knockdown or knockout experiments; dotted black outline of Gb-Dpp orthologs indicates a potentially minimal or absent requirement in PGC specification. Ligand–receptor interactions are schematized only as the specific interactions are unknown for both systems. (B) Phylogenetic distribution of animals for which expression (white circles) or functional (black circles) data suggest that BMP signaling is involved in some aspect of germ cell development (SI Appendix): ESC, conversion of embryonic stem cells to germ cells; GG, gametogenesis; GSC, germ-line stem cells; iPSC, conversion of induced pluripotent stem cells to germ cells; PGCs, primordial germ cells. Red text indicates animals for which functional data support a role for BMP signaling in PGC specification; these data could support a hypothesis of convergent evolution of BMP-based germ cell formation in two or more bilaterian clades. Red diamond indicates the hypothesis of an ancestral role for BMP signaling in bilaterian PGC formation.
would further support the hypothesis that BMP signaling constitutes an ancestral animal mechanism for specification of the germ line.

Materials and Methods

Genes were cloned using sequences from the Gryllus developmental transcriptome (33), with additional sequence data kindly provided by T. Mitu (University of Tokushima, Japan). All sequences reported in this study have been deposited in GenBank under accession nos. KF670859–KF670865. Gryllus culturing, phylogenetic analysis, in situ hybridization, immunostaining, dsRNA synthesis, and qPCR were conducted as previously described (4). Zygotic RNAi was achieved by injection of dsRNA at either 0–5 μl AEL or 24–36 h AEL using previously described injection techniques (4). Small molecule activation of the BMP pathway was accomplished by injecting eggs at 0–5 h AEL with (i) either 10 μM or 10 mM of 4-hydroxyalchone (30) (Santa Cruz Biotechnology Chemicals SC-262260) in DMSO, with DMSO as a control, and (ii) either 10 μg/ml or 100 μg/ml of recombinant Dm-Dpp protein (R&D Systems 159-DP-020/CF) in dilute HCl as per the manufacturer’s instructions, with 10 or 100 μg/ml of BSA in dilute HCl as a control. To quantify PGCs, the volume of each PGC cluster at 4 d AEL was calculated as an ellipsoid with confocal micrographs of optical sections through the region of the abdomen containing germ cells (SI Appendix, Fig. S4). The volume of each PGC in a cluster was both independent of the number of PGCs in a cluster and unaffected by BMP RNAi treatments. The calculated volume of a whole PGC cluster was thus strongly positively correlated with the number of PGCs in that cluster. Measurements of PGC cluster volume, which were more efficient than directly counting PGCs, therefore allowed us to accurately determine the total number of PGCs per cluster, per segment, and per embryo.

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