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# Myo/Nog cell regulation of bone morphogenetic protein signaling in the blastocyst is essential for normal morphogenesis and striated muscle lineage specification

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ABSTRACT

Cells that express MyoD mRNA, the G8 antigen and the bone morphogenetic protein (BMP) inhibitor noggin (Nog) are present in the epiblast before gastrulation. Ablation of "Myo/Nog" cells in the blastocyst results in an expansion of canonical BMP signaling and prevents the expression of noggin and follistatin before and after the onset of gastrulation. Once eliminated in the epiblast, they are neither replaced nor compensated for as development progresses. Older embryos lacking Myo/Nog cells exhibit severe axial malformations. Although Wnts and Sonic hedgehog are expressed in ablated embryos, skeletal muscle progenitors expressing Pax3 are missing in the somites. Pax3+ cells do emerge adjacent to Wnt3a+ cells *in vitro*; however, few undergo skeletal myogenesis. Ablation of Myo/Nog cells also results in ectopically placed cardiac progenitors and cardiomyocytes in the somites. Reintroduction of Myo/Nog cells into the epiblast of ablated embryos restores normal patterns of BMP signaling, morphogenesis and skeletal myogenesis, and inhibits the expression of cardiac markers in the somites. This study demonstrates that Myo/Nog cells are essential regulators of BMP signaling in the early epiblast and are indispensable for normal morphogenesis and striated muscle lineage specification.

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## Introduction

Cells of the epiblast undergo dramatic alterations in position, adhesion and gene expression that lead to the development of the primitive streak, establishment of three germ layers during gastrulation and formation of the nervous system. These events are mediated by complex communication networks involving several families of diffusible proteins, including Wnts, Fibroblast Growth Factors and members of the Transforming Growth Factor family (Gilbert, 2010; Stern, 2006). The response of cells to extracellular factors depends, in part, on where they lie within the concentration gradient and the presence of inhibitors of signal transduction (Ashe and Briscoe, 2006).

In the chick embryo, the first visible sign of primitive streak formation is an increase in cell density in the posterior/medial, stage 2 epiblast (Bellairs, 1986). A hallmark of primitive streak formation is the disappearance of bone morphogenetic protein 4 (BMP4) and its signal transducer, phosphorylated Smad1/5/8 (p-Smad1/5/8), from this region of the epiblast (Faure et al., 2002; Streit et al., 1998; Wilson et al., 2000). The downregulation of BMP signaling in the epiblast was attributed to suppression of BMP4 gene transcription by FGF3 (Wilson et al., 2000) and blocking BMP receptor activation by chordin (Streit et al., 1998). Two other BMP inhibitors, noggin and follistatin, were not detected in the epiblast of the prestreak chick embryo (Chapman et al., 2002; Streit et al., 1998; Wilson et al., 2000); however, both of these BMP inhibitors are expressed in the early *Xenopus* embryo (Khokha et al., 2005; Wessely et al., 2001). Once gastrulation begins, noggin, follistatin, chordin and other BMP inhibitors are synthesized in an important signaling center located at the anterior end of the primitive streak called Hensen's node in the chick, the node in mammals and Spemann's organizer in amphibians (Anderson et al., 2002; Bachiller et al., 2000; Bouwmeester et al., 1996; Capdevila and Johnson, 1998; Chapman et al., 2002; Glinka et al., 1998; Hansen et al., 1997; Hemmati-Brivanlou et al., 1994; Hsu et al., 1998; Khokha et al., 2005; Klingensmith et al., 1999; Lamb et al., 1993; McMahon et al., 1998; Sasai et al., 1994; Smith and Harland, 1992; Streit et al., 1998; Wessely et al., 2001).

The roles of BMP inhibitors in multiple developmental processes have been explored, in part, by adding them to embryonic tissues and by molecular knockout or knockdown of their expression. While addition of noggin or chordin to the embryo induces the formation of ectopic primitive streaks in chick and *Xenopus* embryos (Frisch and Wright, 1998; Graff et al., 1994; Hawley et al., 1995; Streit et al., 1998; Streit and Stern, 1999b; Suzuki et al., 1997), gastrulation does occur in

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noggin and chordin single and double homozygous mutant mice (Anderson et al., 2002; Bachiller et al., 2000; Brunet et al., 1998; Klingensmith et al., 1999; McMahon et al., 1998; Stottmann et al., 2006). BMP inhibitors were also shown to induce neural tissue in Xenopus embryos, maintain the expression of neural markers, establish the border between neural and epidermal tissues in the chick embryo, and regulate morphogenesis and patterning of the mouse neural tube (Anderson et al., 2002; Harland and Gerhart, 1997; Hartley et al., 2001; Khokha et al., 2005; Linker and Stern, 2004; Lumsden and Krumlauf, 1996; McMahon et al., 1998; Sasai et al., 1994; Smith and Harland, 1992; Stottmann et al., 2006; Streit and Stern, 1999a; Streit et al., 1997, 1998; Tanabe and Jessell, 1996; Wills et al., 2010). Chordin null mice exhibit defects in the development of the skeleton, ear, pharynx and cardiovascular system (Bachiller et al., 2000), whereas the absence of follistatin results in musculoskeletal defects and abnormalities in whisker, tooth and skin development (Matzuk et al., 1995). Knockout of noggin in the mouse embryo results in neural tube patterning and fusion defects, enlargement of the notochord, malformations of the heart, lungs, skeleton and esophagus, and a decrease in skeletal muscle in the somites (Anderson et al., 2002; Bachiller et al., 2000; Brunet et al., 1998; Choi et al., 2007; McMahon et al., 1998; Que et al., 2006; Stottmann et al., 2006; Weaver et al., 2003).

A source of noggin in the chick embryo during the period of organogenesis are cells that also express mRNA for the skeletal muscle specific transcription factor MyoD and the cell surface G8 antigen (Gerhart et al., 2006, 2009). These cells arise in the early epiblast and are integrated into all three germ layers during gastrulation (Gerhart et al., 2000, 2006; Strony et al., 2005). Later they are found in multiple tissues and organs, including the somites, eyes, heart and central nervous system (Gerhart et al., 2001, 2006, 2009). The importance of MyoD-positive (+) epiblast cells as a noggin delivery system in the eyes and somites was demonstrated by ablating them during early stages of primitive streak formation. Ablated embryos exhibited eye defects, a herniation of organs through the ventral body wall and a severe reduction in skeletal muscle (Gerhart et al., 2006, 2009). Myogenic progenitor cells expressing the Pax3 transcription factor were present in the somites of ablated embryos; however, they were unable to differentiate unless they were exposed to exogenous noggin (Gerhart et al., 2006).

In the following study we examined whether MyoD+ cells express noggin while they reside within the epiblast and defined the consequences of ablating them in the blastocyst prior to the formation of the primitive streak. Noggin producing, MyoD + (Myo/Nog) cells regulate BMP signaling and follistatin expression during the hours leading up to primitive streak formation. The double noggin/follistatin knockdown resulting from the ablation of Myo/Nog cells in the blastocyst has profound consequences on development that includes a disruption in neurulation, an inhibition of the emergence of skeletal muscle progenitor cells and ectopic cardiomyogenic cells in the somites.

## Materials and methods

### *Immunofluorescence localization and in situ hybridization*

White Leghorn chick embryos (BE Eggs, York, PA) were staged according to the method of Eyal-Giladi and Kochav (1976) (stages X–XII) or Hamburger and Hamilton (1951) (stage 2 and older embryos). Whole stage X-4 embryos and 10 μm paraffin sections from 3 to 6 day embryos were double labeled for the G8 antigen and mRNAs by incubating with the G8 IgM MAb and DyLight 488 conjugated goat anti-mouse IgM μ chain (Invitrogen/Molecular Probes, Eugene, OR), followed by incubation in Cy3 labeled 3DNA™ dendrimers (Genisphere, LLC, Hatfield, PA) conjugated with an antisense cDNA sequence for chicken MyoD (5′-TTCTCAAGAGCAAATACTCAC-CATTTGGTGATTCCGTGTAGTA-3′) (Dechesne et al., 1994) (L34006), chicken noggin (5′-TCTCGTTAAGATCCTTCTCCTTGGGGTCAAA-3′) (Tonegawa and Takahashi, 1998) (NM_204123) or chicken Wnt1 (5′-CAGATCTCGGCCCTTCTCGCTGGAATCCACAAA-3′) (AY655699.1; Jensen, J.L. et al., Direct Submission) (Gerhart et al., 2004a, 2006, 2009). Double labeling was also carried out with IgG MAbs to Pax3 (Venters et al., 2004), sarcomeric myosin heavy chain (MF20 MAb) (Bader et al., 1982), neurofilament associated antigen (3A10 MAb) (Furley et al., 1990), the notochord marker NOT1, cardiac troponin I (TI-1 MAb) (Saggin et al., 1989), Shh (5E1 MAb) (Ericson et al., 1996) (all obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA), MyoD1 (NCL-MyoD1 MAb from Vector Laboratories, Burlingame, CA), BMP2 (Sigma-Aldrich, St. Louis, MO) and cardiac troponin T, isoform Ab-1 (MS-295-PO, Thermo Scientific, Fremont, CA), and the following polyclonal antisera: goat anti-mouse noggin (AF719, R&D Systems, Minneapolis, MN), rabbit anti-mouse chordin (ab24562, Abcam, Cambridge, MA), goat anti-human follistatin (sc-23553, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human BMP4 (5674R-100, Biovision, Mountain View, CA), rabbit anti-human p-Smad1/5/8 (9511, Cell Signaling Technology, Danvers, MA), rabbit anti-human Wnt3a (ab28472, Abcam), rabbit anti-human GATA4 (ab25992, Abcam) and rabbit anti-human Smad4 (sc-7154, Santa Cruz Biotechnology). IgG MAbs were tagged with affinity purified, goat anti-mouse IgG conjugated with rhodamine (Jackson ImmunoResearch, West Grove, PA) and fluorescein or rhodamine labeled, rabbit anti-mouse IgG2b or IgG1 (Fitzgerald Industries, Inc., Concord, MA). Polyclonal antibodies were labeled with donkey anti-goat and goat anti-rabbit IgG conjugated with fluorescein, DyLight 488 or DyLight 549 (Jackson ImmunoResearch, Chemicon and Invitrogen/Molecular Probes). Nuclei were counterstained with Hoechst dye 33258 (1 μg/ml Hanks buffer) (Sigma-Aldrich). Experiments included sections stained with secondary antibodies alone to determine the level of background fluorescence.

Embryos and sections were mounted in Gelmount (Biomeda, Foster City, CA) or Elvanol (Dupont, Wilmington, DE) and analyzed with a Nikon Eclipse E800 epifluorescence microscope (Optical Apparatus) equipped with 4× NA 0.2, 40× oil NA and 60× oil NA 1.4 objectives and the following filters: excitation 530–560, barrier 573–648 for Cy3 and Rhodamine; excitation 465–495, barrier 515–555 for Alexa 488; excitation 330–380, barrier 435–485 for Hoechst dye. Images were captured and produced with the Evolution QE Optronics video camera (Media Cybernetics) and Image Pro Plus image analysis software program (Phase 3 Imaging Systems). Figures were annotated and adjusted for brightness and contrast using Adobe Photoshop 6.0.

### *Ablating cells in the epiblast and incubation of embryos*

MyoD+ cells were ablated in stage X–XII epiblasts by incubating embryos with the G8 MAb and lysing labeled cells with baby rabbit complement (Cedar Lane, Inc., Hornby, Ontario, Canada) (Gerhart et al., 2006, 2008, 2009). For these studies, antibody and complement solutions were applied to the vitelline membrane as the embryo resided on the yolk (Gerhart et al., 2008). Similar numbers of cells bound the G8 MAb whether the solutions were applied to isolated embryos or embryos within the vitelline membrane. Control embryos were incubated in Hanks buffer, complement only or the D4 MAb and complement. Hybridoma cells producing the D4 MAb were generated by immunizing BALB/c mice with three inoculations of $10^6$ cells obtained from the posterior 12 pairs of somites and segmental plate mesoderm of stage 12–14 chick embryos, as described previously for the generation of the G8 MAb (Gerhart et al., 2001). Cell lysis and death were visualized by incubating embryos in trypan blue (Gerhart et al., 2006) and staining for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche Diagnostics, Mannheim, Germany). Control and ablated embryos were incubated on the yolk

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