TECHNOLOGY REPORT

RNA Inhibition of BMP-4 Gene Expression in Postimplantation Mouse Embryos

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Summary: Short, hairpin RNA (shRNA) directed against bone morphogenetic protein 4 (Bmp-4) was delivered to early postimplantation staged mouse embryos via tail vein injection of pregnant dams. As early as 24 h postinjection, embryos expressed a DsRed marker and later exhibited defects of neural fold elevation and closure and of cardiac morphogenesis. Immunohistochemical analysis of sectioned embryos indicated that Bmp-4 protein was depleted and gene expression analysis indicated there was a reduction in Bmp-4 mRNA and an upregulation of the Bmp-4 antagonists, noggin and chordin, in embryos exposed to the shRNA, but not in control embryos. There was no change in the expression of Gata4, brachyury, or claudin6 in RNAi exposed embryos, indicating that RNA silencing was specific to Bmp-4 rather than producing widespread gene inhibition. Delivery of shRNA to embryos has the potential to specifically knockdown the expression of developmentally essential genes and to rescue gene mutations, significantly decreasing the time required to analyze the function(s) of individual genes in development. genesis 37:12–17, 2003. © 2003 Wiley-Liss, Inc.

Key words: bone morphogenetic protein 4; development; embryo; gene expression; gene targeting; mouse; RNAi

Gene targeting in the mouse embryo has produced tremendous insights into gene function during development (Doetschmann *et al.*, 1987; Thomas and Capecchi, 1987). Although gene deletion is considered to be the gold standard, generation of targeted mutant mice is a tedious process and problematic if the targeted gene produces an embryonic lethal phenotype. Other approaches such as using morpholino antisense oligonucleotides have also been developed to disrupt gene expression in embryos, but have not been widely employed because of problems with toxicity, nonspecific effects, and the short life of the morpholino (Siddall *et al.*, 2002). Over the last few years, there have been tremendous advances in sequence-specific gene silencing using RNA interference techniques (Paddison and Hannon, 2002). The phenomenon was identified in *Caenorhabditis elegans*, where it was noted that the control RNA (sense RNA) also downregulated gene expression. It was initially thought that this was an aberrant cellular event, but it is now clear that this process is an innate method employed by many organisms to control gene expression. In addition, RNA interference is now recognized as a powerful tool to produce gene silencing. When introduced into cells, small interfering RNAs (siRNA), or RNAs expressed as short hairpins (shRNA), efficiently target and cause degradation of the cognate RNAs (Paddison and Hannon, 2002).

Posttranscriptional gene silencing by RNAi has been employed extensively in *C. elegans* (Fire *et al.*, 1998) and more recently in mammalian cells (Elbashir *et al.*, 2001; Brummelkamp *et al.*, 2002; Yang *et al.*, 2001, 2002). siRNAs have been expressed in mouse embryos by electroporation or injection of preimplantation staged embryos (Grabarek *et al.*, 2002; Wianny and Zernicka-Goetz, 2002), and in postimplantation embryos via exo-utero surgery and regional electroporation (Calegari *et al.*, 2002). Transgenic expression of siRNA has also recently been achieved by electroporation of ES cells followed by chimera formation (Carmell *et al.*, 2003; Rubinson *et al.*, 2003), or following tetrapoloid aggregation (Kunath *et al.*, 2003), which produced embryos that expressed shRNA to Rasa 1 and phenocopied the Rasa1 null mouse. Others have injected fertilized eggs with shRNA plasmids (Hasuwa *et al.*, 2002) or transduced two-cell embryos with lentiviral vectors expressing shRNAs (Tiscornia *et al.*, 2003) and observed stable, heritable gene knockdown. RNA interference has the advantages that individual genes or combinations can be targeted, multiple regions of the same transcript can be targeted, and the RNAi can be constitutively expressed to produce long-term gene suppression. If delivered to pregnant animals, the length of time required to assess gene function could be greatly reduced compared with conventional germ line mutagenesis strategies. Unlike

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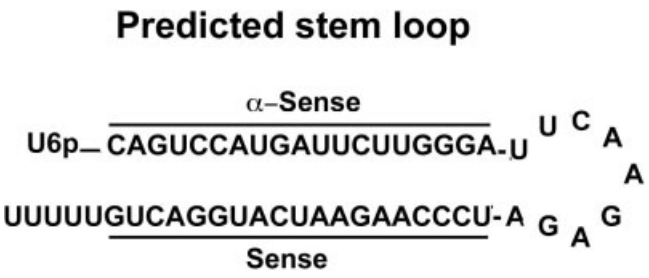

FIG. 1. Schematic of the predicted hairpin structure containing the antisense and sense regions that target the Bmp4 gene, and a 9 bp loop.

gene targeting using siRNA in ES cells, this approach allows targeting of genes expressed in the trophoblast and yolk sac—structures that are not normally formed from ES cells, as well as genes involved in growth and maintenance of the ES cells themselves. In addition, the timing of the delivery of the shRNA can be precisely controlled to target the stage of interest, making it a powerful approach to study genes whose loss of function produces early embryonic lethality. Finally, different levels of RNA silencing (Hemann *et al.*, 2003) produced by targeting different regions of the transcript may be effective in generating hypomorphic alleles and in teasing out gene structure/function relationships.

In this investigation, we delivered shRNA targeted to bone morphogenetic protein 4 (Bmp-4) mRNA (Fig. 1) to early postimplantation staged mouse embryos using tail vein injection. Rather than coinjection of a reporter plasmid with separate siRNAs (Calegari *et al.*, 2002; Miyagishi and Taira, 2002; Ya *et al.*, 2002), a single vector was designed in which DsRed was expressed from the CMV promoter and constitutive expression of Bmp-4i was controlled by the mouse RNA polymerase III U6 promoter (Miyagishi and Taira, 2002; Ya *et al.*, 2002). Unlike high-pressure tail vein injection designed to open capillaries and deliver an siRNA to adult tissues (Lewis *et al.*, 2002), we employed low-pressure injection to deliver the shRNA, with the goal of examining the effects of Bmp-4 gene knockdown during early postimplantation stages of embryonic development.

Embryonic day (E) 6.5 time pregnant mice were injected via the tail vein with either pCS2/Red (control) or pCS2/Red/Bmp-4i (pBmp4i), and sacrificed after 24 h, 48 h, 72 h, and 8 days (E14.5) postinjection. As early as 24 h postinjection, embryos expressed DsRed (Fig. 2A), with expression persisting over the next 72 h, extending to E14.5 (Fig. 2C). Within an individual litter, there was some variation between embryos in their expression of the DsRed marker: a majority (64%) of the embryos examined expressed DsRed with no preference for a particular day of gestation. Controls expressed DsRed within all tissues in the embryo (Fig. 2G) and no morphological abnormalities were observed in these embryos. Bmp-4i exposed embryos exhibited a number of morphological alterations, including defects of neural tube closure consisting of a failure of neural fold elevation in the cephalic region (Fig. 2B), rachischisis, a truncated posterior axis, and occasionally a small allantois. The first branchial arch was often hypoplastic and defects of cardiac looping were also evident. These phenotypes appeared to reflect hypomorphic behavior of the lethal phenotype previously observed in germline-targeted Bmp-4 null embryos (Fujiwara *et al.*, 2001, 2002; Hebert *et al.*, 2003). It would be of considerable interest to examine primordial germ cell differentiation in these embryos since the expression of genes such as fragilis that are involved in specification of primordial germ cells also appear to be affected by Bmp-4 expression levels (Lawson *et al.*, 1999; Saitou *et al.*, 2002). Interestingly, maternal tissues expressed variable levels of the DsRed marker, as observed previously (Lewis *et al.*, 2002). There was constant high expression in liver, pancreas, spleen, and heart, and variable expression in skin and striated muscle, while brain never expressed DsRed.

To determine if Bmp-4 protein expression was affected in the shRNA exposed embryos, immunohistochemical analyses were carried out on sectioned embryos (Fig. 3). Unlike control embryos that expressed high levels of Bmp-4 in the surface ectoderm (Fig. 3E,F), there was very little Bmp-4 protein in the shRNA-exposed embryos (Fig. 3A–D). Scattered Bmp-4 immunoreactivity was present in the surface ectoderm of embryos on E8.5 and E9.5; by E14.5 there was little expression in the CNS, lung, or epidermal ectoderm. The function of the Bmp-4 hairpin was also assessed by comparison of Bmp-4 expression in control (pCSRed) and pBmp-4i embryos using RT-PCR. The expression of Bmp-4 mRNA was diminished 4–8-fold in embryos that received the Bmp-4i cassette (Fig. 4, lane 1) compared to pCSRed control embryos. Importantly, expression of the DsRed or Bmp-4i cassettes did not affect global transcriptional regulation as expression of an endoderm marker Gata4 (Fig. 4, lane 6), Brachyury (T), a mesoderm marker (Fig. 4, lane 7), claudin6, an ectoderm marker (Fig. 4, lane 8), and β-actin, a positive control (Fig. 4, lane 9), were similar in both groups, indicating that the shRNA silenced Bmp-4 specifically rather than producing widespread gene inhibition. Interestingly, mRNA for the Bmp-4 antagonists, chordin and noggin, was upregulated in Bmp-4i exposed embryos (4-fold and 12-fold, respectively) (Fig. 4, lanes 2 and 3). Musashi1, a marker of primitive neuroepithelium, was strikingly upregulated in the presence of Bmp-4i by 30-fold (Fig. 4, lane 4). Nodal, a downstream target of Bmp-4 was unexpectedly upregulated 3-fold (Fig. 4, lane 5), possibly releasing control over L- R-asymmetry in the embryo.

These results demonstrate for the first time the systemic delivery and expression of shRNA directed to a gene critical in the development of postimplantation mouse embryos. The results phenocopy many of the characteristics of the Bmp-4 gene targeted embryos and identify alterations in the expression of target genes. Although it is not yet known if this approach will be

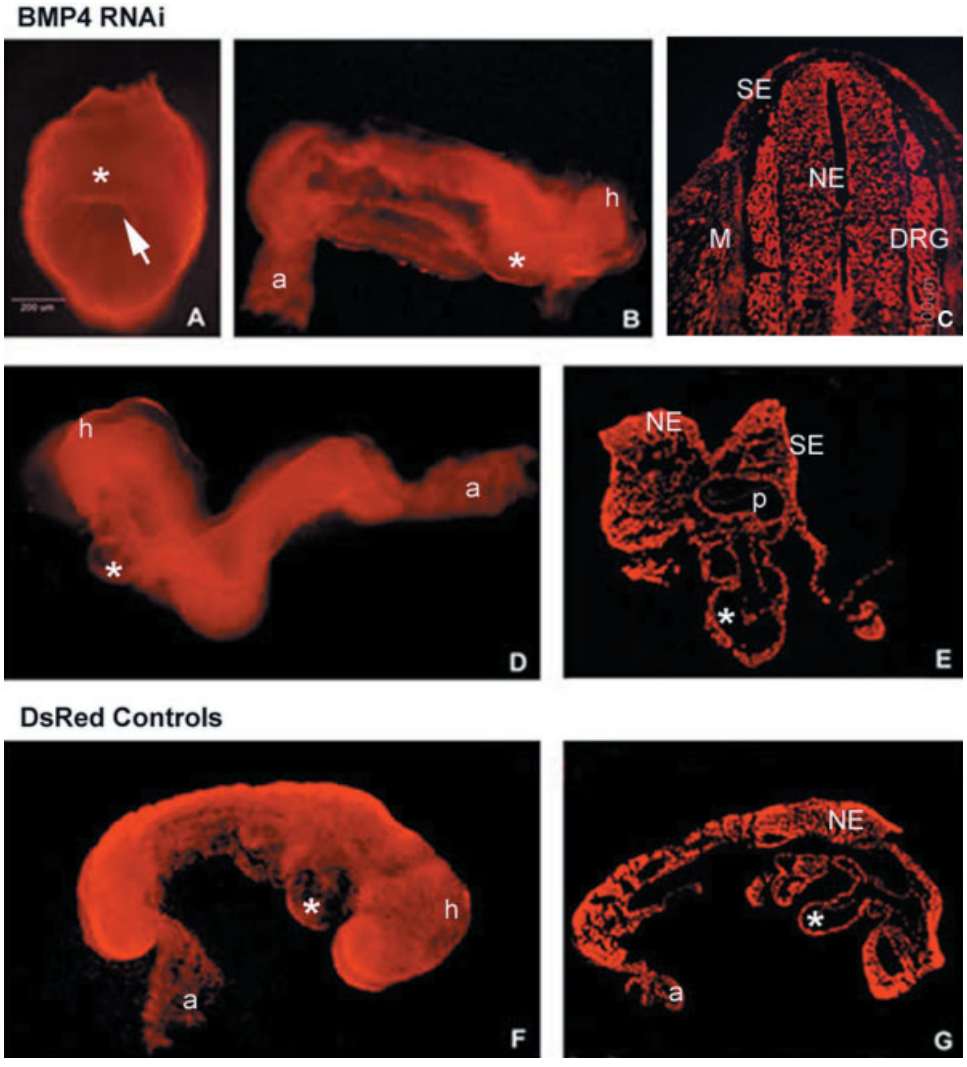

FIG. 2. Embryos were isolated on E7.5 (**A**), E8.5 (**B,D–G**), or E14.5 (**C**) of gestation from pregnant females injected on gestation E6.5 with Bmp-4i (**A–E**) or DsRed control plasmid (**F,G**). As early as 24 h postinjection, the DsRed marker was expressed in E7.5 embryos (**A**) and was uniformly distributed throughout sectioned embryos examined on E14.5 (**C**) or on E8.5 (**E,G**). Embryos exposed to Bmp-4i exhibited defects of neural fold elevation and closure (**B**), turning and cardiac rotation (**B,D,E**), compared with morphologically normal control embryos (**F,G**). Asterisks indicates the cardiac region; arrow in **A**, the foregut pocket. a = allantois; h = headfolds; SE = surface ectoderm; NE = neuroepithelium; M = forming myotome; DRG = dorsal root ganglion; p = pharynx.

successful later in development after placental development is more complete, this potential limitation might be overcome by early injection of plasmids in which expression of the RNAi is controlled by an inducible promoter. The use of RNAi to study gene function in development has many advantages including: precise control of gene knockdown at a specific stage of embryonic development, the ability to target multiple genes without complicated breeding regimens, to attempt gene rescue, and in the long term to treat genetic, prenatal conditions.

MATERIALS AND METHODS

Construction of cDNA Expression Vectors

A BamHI/XbaI fragment that contained the DsRed coding region was removed from pDsRed2-1 (ClonTech, Palo Alto, CA) and ligated into the first MCS of pCS2 (Turner and Weintraub, 1994) to generate pCS2/Red. The mouse U6 promoter (GenBank X06980) was PCR-amplified from genomic DNA and cloned into the EcoRI and BamHI sites in pBS SK (Stratagene, La Jolla, CA) to yield pSKU6. An RNAi hairpin was designed to target nt 305–323 of the Bmp-4 mRNA (GenBank X56848) and ligated downstream of the U6 promoter in pSKU6 (Fig. 1). The final vector pCS2/Red/Bmp-4i was constructed by removing the U6/BMP-4i cDNA from the SK using NotI and KpnI followed by ligation of this cDNA into the second MCS of pCS2/Red to yield pCS2/Red/Bmp-4i (pBmp-4i). Plasmid preparations for injections were prepared with the Qiagen (Chatsworth, CA) Maxi Prep Kit.

Tail Vein Injections

Plasmids (10 μg) were diluted in Ringer's solution and injected into the tail vein of unanesthetized, time pregnant (E6.5; plug day = E0.5) mice (CD-1, Charles River, Portage, MI) in a volume of 250 μl, using a 23 G butterfly needle and a 1 cc syringe. At least 10 mice were injected per replicate experiment (n = 4 replicates). Over 398 embryos from a total of 32 pregnant females were ex-

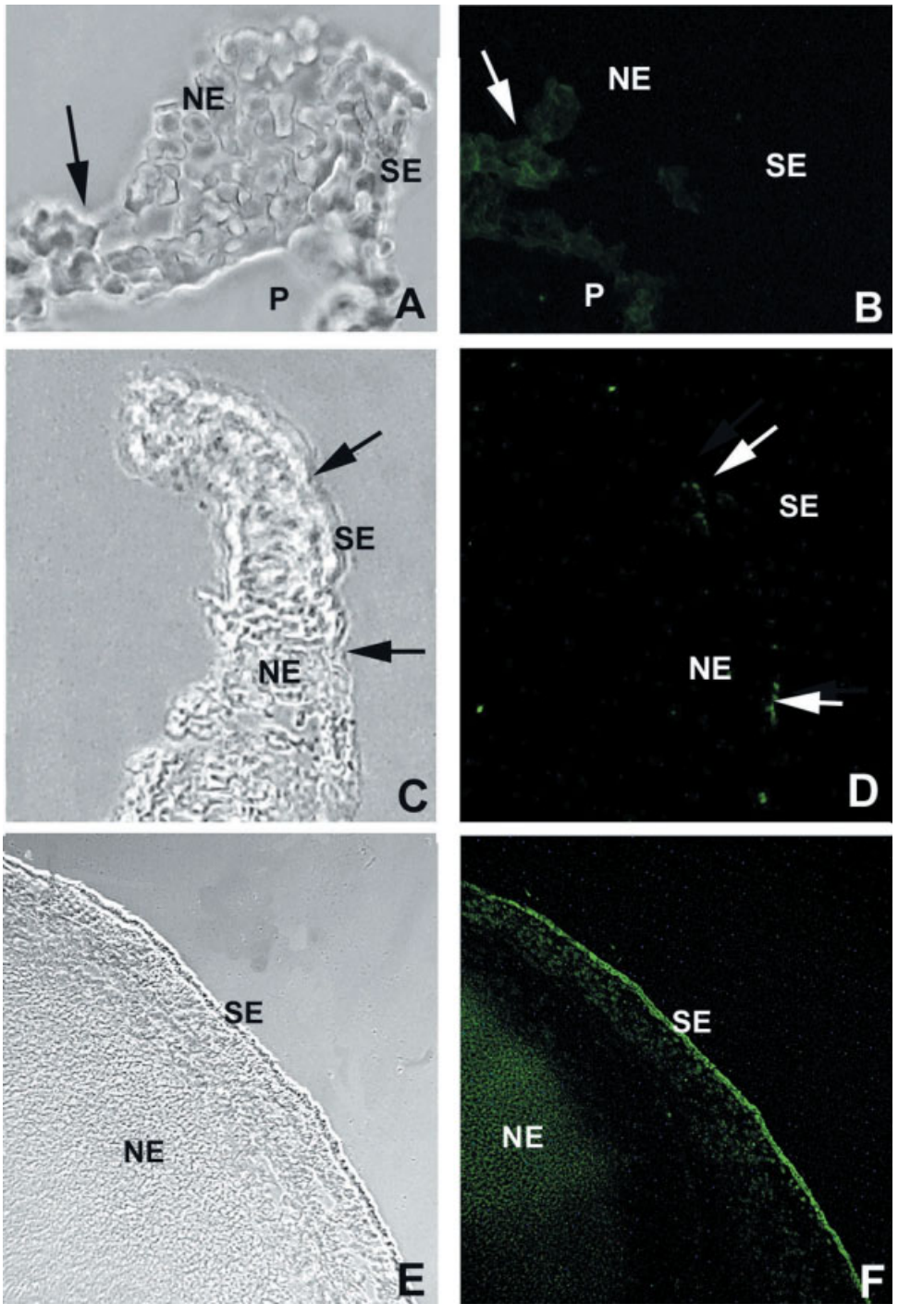

FIG. 3. Immunohistochemical localization of Bmp-4 protein in embryos exposed to pBmp-4i (**A-D**), and DsRed control embryos (**E,F**). Phase contrast micrographs (**A,C,E**) illustrate the organization of the surface ectoderm (SE) and underlying neuroepithelium (NE). The neural folds are collapsed in this early somite embryo (E8.5, **A,B**) obliterating much of the mesenchyme. There was no immunoreactivity for Bmp-4 in the surface ectoderm, where it is normally expressed; with slight expression in the midline (arrow) and in the endoderm surrounding the pharynx (P). By E9.5 (**C,D**), the neural folds had not yet closed in many of the Bmp-4i-exposed embryos, and there was only slight scattered staining of Bmp-4 (arrows) in the surface ectoderm (SE). Control embryos exhibited normal neural tube closure and strong immunoreactivity for Bmp-4 in the surface ectoderm (**E,F**).

amined. Mice were sacrificed at 24 h, 48 h, 72 h, and 8 days (E14.5) postinjection. Embryos were removed from the uterus and membranes and examined using epifluorescence microscopy to determine the extent of DsRed expression. Embryos were then either processed for histological analysis, SEM, or RNA extraction. Additional maternal tissues: spleen, liver, kidney, lung, heart, striated muscle, skin, and brain were removed 72 h postinjection and embedded in OCT. Frozen sections were cut and examined using epifluorescence to determine the pattern of expression of the DsRed marker.

Immunohistochemical localization of Bmp-4 protein was carried on 10-μ thick frozen sections of embryos. Sections were fixed briefly, washed, blocked, then exposed to anti-Bmp-4 antibodies (1:50, Santa Cruz Biotechnology, sc-6896, Santa Cruz, CA) followed by sec-

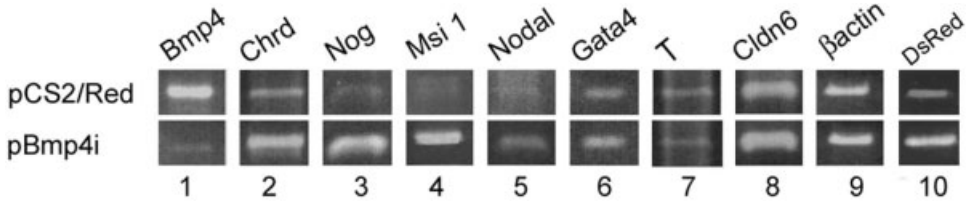

FIG. 4. RT-PCR analysis of gene expression in pCS2/Red control and pBmp-4i-exposed embryos. RNA was isolated from embryos on E8.5 of gestation and RNA (1 μg) was reverse-transcribed in the presence of oligo-dT and PCR with gene-specific primers was performed as described in Materials and Methods. The PCR products were electrophoresed in 1.5% agarose ethidium bromide gels. Expression of Bmp-4 decreased following exposure to Bmp-4i (lane 1). The expression of the Bmp-4 antagonists, chordin and noggin, as well as musashi1 and nodal, are upregulated in the Bmp-4i embryos (lanes 2–5). Gata4, an endoderm marker, Brachyury (T), a marker of mesoderm, Claudin 6 (cldn6), a marker of early epidermal ectoderm, and β-actin expression were unchanged between controls and pBmp-4i-exposed embryos (lanes 6–9). DsRed expression was slightly higher in Bmp-4i embryos compared with controls (lane 10).

ondary antibodies conjugated to FITC. Sections were examined and photographed using epifluorescence microscopy and digital images imported to PhotoShop (Adobe Systems, Mountain View, CA) to construct illustrations.

RT-PCR Analysis of Gene Expression

RNA was extracted from E8.5 and E9.5 embryos with Trizol (ILT), DNAsed, and quantified. Approximately 10–12 embryos were recovered per litter and combined in a single Trizol aliquot for extraction. One control and two Bmp-4i-exposed litters were examined for each experiment. The RT-PCR was done in triplicate for each embryo pool. The RNAs (1 μg) served as templates in oligo dT primed reverse transcription (RT) reactions, then 1/10 of the reaction was used as a template in PCR with gene-specific primers. The optimal number of PCR cycles was determined for each primer pair to ensure amplification in the linear range (Gratsch, 2002). General PCR conditions were 94°/3 min, 94°/30 sec–1 min, 51–60°/1 min, 72°/2 min for 25–35 cycles, and a final extension of 72/7 min.

PCR primers.

β-actin, F-aaccctaaggccaaccgtg, and R-caggattccatacccaagaagg (498 bp, 22 cycles); Bmp-4, F-ctcccaagaatcatggactg and R-aaagcagagctctcactggt (468 bp, 32 cycles); noggin, F-cccggtgctgtacgcgtgg and R-gcaggaacacttacactcg (250 bp, 29 cycles); chordin, F-accaacgcagtagagacctccc and R-ggggtagcaggaatggtgtg (901 bp, 30 cycles); DsRed, F-tcaccgagttcatgcgcttca and R-cgttgtgggaggtgatgtccagct (593 bp, 30 cycles); Musashi 1, F-ttccaagccacgacctacgc and R-ggagcgagagaagagacaaggtctg (638 bp, 30 cycles); nodal, F-atttgccagacagaagccaac and R-tcctccacaatcatgtccttg (312 bp, 29 cycles); Brachyury, F-agaaagaaacgaccacaaagatg and R-atttatttatttttcccttgtcc (729 bp, 33 cycles); Claudin 6, F-gataggaactccaagtctcgt and R-tgggacagatgtagaatagca (287 bp, 29 cycles); Gata4, F-ccgagcaggaatttgaagagg and R-gcctgtatgtaatgcctgcg (469 bp, 35 cycles). The PCR products were electrophoresed in 1.5% agarose/EtBr gels and photographed in a UV light box. For semiquantitative analyses, the gels were scanned, then analyzed using a Lab Works 4.0 (Cybergenetics, Pittsburgh, PA) densitometry program.

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