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Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure

Keywords: Bisphenol A; *In vitro*; *In vivo*; Rat; Mouse; Aquatic animal; Cancer; Low dose; Non-monotonic dose–response curves; Developmental programming

1. Introduction

This document is a summary statement of the outcome from the meeting: "*Bisphenol A: An Examination of the Relevance of Ecological, In vitro and Laboratory Animal Studies for Assessing Risks to Human Health*" sponsored by both the NIEHS and NIDCR at NIH/DHHS, as well as the US-EPA and Commonweal on the estrogenic environmental chemical bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane; CAS# 80-05-7). The meeting was held in Chapel Hill, NC, 28–30 November 2006 due to concerns about the potential for a relationship between BPA and negative trends in human health that have occurred in recent decades. Examples include increases in abnormal penile/urethra development in males, early sexual maturation in females, an increase in neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD) and autism, an increase in childhood and adult obesity and type 2 diabetes, a regional decrease in sperm count, and an increase in hormonally mediated cancers, such as prostate and breast cancers. Concern has been elevated by published studies reporting a relationship between treatment with "low doses" of BPA and many of theses negative health outcomes in experimental studies in laboratory animals as well as *in vitro* studies identifying plausible molecular mechanisms that could mediate such effects. Importantly, much evidence suggests that these adverse effects are occurring in animals within the range of exposure to BPA of the typical human living in a developed country, where virtually everyone has measurable blood, tissue and urine levels of BPA that exceed the levels produced by doses used in the "low dose" animal experiments.

Issues relating to BPA were extensively discussed by five panels of experts prior to and during the meeting, and are summarized in five reports included in this issue: (1) human exposure to bisphenol A (BPA) [1]; (2) *in vitro* molecular mechanisms of bisphenol A action [2]; (3) *in vivo* effects of bisphenol A in laboratory animals [3]; (4) an ecological assessment of bisphenol A: evidence from comparative biology [4]; (5) an evaluation of evidence for the carcinogenic activity of bisphenol A [5]. Further discussion occurred at the meeting where participants from the panels were reorganized into four breakout groups. The consensus statements from the meeting are presented below.

The definition of "low dose" of BPA at this meeting used the same two criteria established at a prior NIH meeting concerning the low dose endocrine disruptor issue [6]: (1) for laboratory animal studies "low doses" involved administration of doses below those used in traditional toxicological studies conducted for risk assessment purposes. For BPA the lowest dose previously examined for risk assessment purposes was 50 mg (kg^{-1} day^{-1}) in studies with rats and mice. The 50 mg (kg^{-1} day^{-1}) dose is the currently accepted lowest adverse effect level (LOAEL) that was used to calculate the current US-EPA reference dose (the daily dose that EPA calculates is safe for humans over the lifetime) of 50 μg (kg^{-1} day^{-1}). The current reference dose is thus based on "high dose" experiments conducted in the 1980s [7]. (2) "Low dose" also refers to doses within the range of typical human exposure (excluding occupational exposures). For purposes of this meeting, the published literature that was reviewed met both of these criteria for being considered within the "low dose" range.

Hundreds of *in vitro* and *in vivo* studies regarding the mechanisms and effects of low doses of BPA, as well as studies of biomonitoring and sources of exposure, have been published in peer reviewed journals over the last 10 years, since the first "low dose" BPA *in vivo* studies were published [8–10]. The meeting was convened specifically to integrate this relatively new information. This task required the combined expertise of scientists from many different disciplines, and care was taken to ensure that participants covered these diverse areas.

BPA is a high-volume (>6 billion pounds per year) production chemical used to make resins and polycarbonate plastic [11]. Of particular concern is the use of BPA in food and beverage plastic storage and heating containers and to line metal cans. In addition, potential environmental sources of BPA contamination are due to use in dental fillings and sealants [12], losses at the production site [13], leaching from landfill [14,15], and presence in indoors air [16].

BPA has become a chemical of "high concern" only in recent years, even though BPA was shown to stimulate the reproductive

Abbreviations: ADHD, attention deficit hyperactivity disorder; BADGE, bisphenol A diglycidyl ether; BIS-DMA, bisphenol A dimethacrylate; BIS-GMA, bisphenol A glycerolate dimethacrylate; BPA, bisphenol A; ER, estrogen receptor

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system in female rats and thus to be an "environmental estrogen" in 1936 [17], long before it was used as the monomer to synthesize polycarbonate plastic and resins in the early 1950s. However, more recent evidence has shown that BPA also exhibits other modes of endocrine disruption in addition to binding to estrogen receptors, such as alterations in endogenous hormone synthesis, hormone metabolism and hormone concentrations in blood. BPA also results in changes in tissue enzymes and hormone receptors, and interacts with other hormone-response systems, such as the androgen and thyroid hormone receptor signaling systems. While BPA was initially considered to be a "weak" estrogen based on a lower affinity for estrogen receptor alpha relative to estradiol [18], research shows that BPA is equipotent with estradiol in its ability to activate responses via recently discovered estrogen receptors associated with the cell membrane [19–22]. It is through these receptors that BPA stimulates rapid physiological responses at low picogram per ml (parts per trillion) concentrations.

2. Purpose and organization of the BPA meeting

2.1. Topic-focused expert panels

To address the strength of the evidence regarding the published BPA research, an organizing committee was formed, and five panels of experts from different disciplines were established. Each panel had a chair or co-chairs and included a scientist who agreed to be primarily responsible, along with the chair, for preparing a preliminary draft of the panel's report. A web site was established on which all of the available electronic files of articles concerning BPA were posted, along with other pertinent information relating to the meeting. Prior to the meeting, the panel members began working on draft reports and communicated via electronic media and telephone conference calls. The resulting preliminary report from each panel was posted on the web site and distributed at the meeting for all participants to read. After the meeting, each panel completed a manuscript that is a part of this meeting report. These five panel reports were peer reviewed using the normal manuscript submission process to *Reproductive Toxicology*.The following specific concerns about BPA led to the five expert panels being established:

(1) Leaching of BPA occurs from the resin lining of metal cans and from plastic food and beverage containers under conditions of normal use. BPA is also detected in water and air samples.
(2) Parts per billion (ppb) levels of BPA that are unconjugated (not metabolized and thus biologically active) are detected in human blood and tissues in different countries, and these levels appear to be higher than blood levels that would be present in animals exposed to the US-EPA reference dose.
(3) BPA causes a wide range of adverse effects at "low doses" that are below the US-EPA reference dose in animals, both terrestrial and aquatic.
(4) There is evidence from *in vitro* mechanistic studies that indicates the potential for disruption of human and animal cell function at concentrations of BPA far below unconjugated levels typically found in human blood and tissues.
(5) There is evidence that at very low doses, BPA may be carcinogenic or increase susceptibility to cancer in animals.

The five panels each addressed a different topic related to their specific area of expertise with BPA and prepared a panel report that included documentation of the relevant published studies:

Panel (1) Sources and amounts of human exposure to BPA as well as pharmacokinetics.
Panel (2) *In vitro* studies related to the molecular mechanisms that mediate responses to BPA with an emphasis on studies using low doses.
Panel (3) *In vivo* studies of BPA at "low doses" in laboratory animals.
Panel (4) *In vivo* studies of BPA in aquatic wildlife and laboratory animals.
Panel (5) Relationship of BPA to cancers.

The purpose of the 3-day meeting was to provide an opportunity for members of the different panels to interact with each other to integrate information from different disciplines concerning low dose effects of BPA after each panel of experts had prepared a report in its specific area. The agenda of the meeting was designed to allow the members of the five panels to have time to discuss the information in their panel reports and finalize statements about the strength of the evidence for the literature that the panel had reviewed.

2.2. Integration of information by breakout groups

For the second part of the meeting the focus was on integrating the information from each of the panel reports. This was accomplished by assigning panel members to one of four breakout groups. The four replicate breakout groups were established using the following criteria, such that each breakout group should have

(1) At least two members from each of the five panels.
(2) A person from each panel who had published on BPA.
(3) A person with general knowledge of endocrine disruption research or endocrinology, but who had not necessarily published on BPA.
(4) A person with experience in the process of reaching consensus.
(5) A mixture of junior and senior investigators.

The charge to the replicate breakout groups was to individually integrate the information relating to the following four issues:

Issue (1) Determine the degree to which the findings on BPA mechanisms of action identify mechanisms and bioactive doses that explain results of the studies reported by the panel on *in vivo* laboratory animal studies. Determine the strength of the evidence for plausible mechanisms mediating *in vivo* effects at low doses. In

addition, identify any *in vivo* findings that are unexpected based on the *in vitro* literature.

Issue (2) Assess the degree to which ecological studies with wildlife are consistent with laboratory studies in similar and different species. For example, determine the similarity of exposure levels and types of responses seen in wildlife and laboratory animals.

Issue (3) Discuss the degree to which the low doses of BPA used in laboratory animal studies relate to the levels detected in human serum and tissues (including urine).

Issue (4) Assess the importance of life stage in the pharmacokinetics of BPA, levels of exposure to BPA, and the health effects of BPA in animals and humans.

3. Findings submitted by the four breakout groups

The reports from the breakout groups are presented below. The four breakout groups conducted a critical examination of the published research on BPA in relation to the four topics described above. Each of the breakout groups identified areas of knowledge and research gaps and made suggestions for future directions of research. In addition, each group identified which of the following two categories applied to specific outcomes:

- "We are confident of the following": this category applied when there were findings reported in multiple papers from multiple labs that were in agreement. There should have been no papers reporting conflicting findings, unless there were flaws in those papers, in which case the flaw(s) should have been identified.
- "We believe the following to be likely but requiring confirmation": This category applied when there were multiple consistent findings from one lab, or there may have been some conflicting reports along with reports of significant findings.

4. Levels of confidence for published BPA findings

The responses from the four different breakout groups were integrated together and organized based on levels of confidence. The criterion for a statement being included in a category was that there had to be consensus among all four of the breakout groups about the statement.

4.1. Based on existing data we are confident of the following

4.1.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. *In vitro* studies have provided two routes of plausibility for low dose *in vivo* effects of BPA. These include binding to nuclear estrogen receptors that regulate transcription as well as estrogen receptors associated with the cell membrane that promote calcium mobilization and intracellular signaling. Receptors associated with the cell membrane are more sensitive to BPA than the nuclear receptors. Actions mediated by membrane associated receptor signaling may underlie much of the low dose BPA phenomena (effects have been reported at doses as low as 1 pM or 0.23 ppt). This increases the plausibility of effects at low doses, which are within the range of environmentally relevant doses (human and wildlife levels of exposure).
2. *In vitro* mechanistic information has informed us that exposing tissues to only an extremely narrow range of doses of BPA may lead to erroneous conclusions. Non-monotonic dose–response curves are encountered frequently in basic endocrinological research, and numerous examples have been reported for BPA reviewed in Refs. [18,23,24]. Because of this animal experiments on unstudied systems must avoid narrow dose ranges, especially the use of only a few very high doses. Thus, testing one or two doses and concluding that there are no effects is inappropriate. At somewhat higher doses than are required for estrogen receptor (ER)-mediated responses, BPA also interacts with androgen and thyroid hormone receptors, making predictions of effects at different doses very complex.
3. *In vitro* studies can dissect mechanisms of complicated effects observed *in vivo*. The proposed potential mechanisms acting *in vitro* and *in vivo* are the same, involving estrogen receptor mediated (nuclear- and membrane-associated) actions. However, specific effects are dose and cell/tissue specific. In addition, there are *in vivo* processes that are not reflective of currently known mechanisms that have been identified *in vitro*. This is due to previously unknown mechanisms as well as the complexity (due to interactions among cell and tissue types) of *in vivo* systems.

4.1.2. Issue 2: Wildlife—laboratory animal research connection

1. BPA is found in the environment: aquatic, terrestrial and air.
2. Studies of wildlife demonstrate estrogenic responses that are similar to responses seen in laboratory animals. Specifically, reductions in spermatogenesis are seen in wildlife at ecological concentrations of BPA, and these effects are also seen in controlled laboratory studies with BPA. In addition, vitellogenin response is a common biomarker in non-mammalian wildlife and laboratory species for BPA-induced estrogen receptor activation as well as activation by other estrogens.
3. BPA exposure induces similar effects in reproductive systems in wildlife and experimental animal model systems, but concentrations used in experiments involving wildlife species are often higher than environmental exposures. There are conditions in the environment, such as landfill leachates and effluent outflow that cause episodic exposure of field populations to elevated doses of BPA.
4. Responses in a variety of vertebrate wildlife species are qualitatively consistent with controlled laboratory studies with BPA. Thus, animals in the wild show evidence of harm, and controlled laboratory studies with model aquatic animals (i.e., medaka, zebrafish, and fathead minnows) are consistent with observations made in wildlife species. Low dose effects of BPA (low ppb range) have been observed in many of these animals.

5. The similar effects observed in wildlife and laboratory animals exposed to BPA predict that similar effects are also occurring in humans.

4.1.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure to BPA is widespread.
2. Human exposure to BPA is variable, and exposure levels cover a broad range [central tendency for unconjugated BPA: 0.3–4.4 ng ml^{-1} (ppb)] in tissues and fluids in fetuses, children and adults.
3. Because the current published literature states that there is a linear relationship between administered dose and circulating levels of BPA in animal studies, this allows circulating levels at lower administered doses to be predicted in experimental animals based on the results from studies in which higher doses were administered.
4. All of the currently published metabolic studies in rats predict circulating BPA levels after acute low dose oral exposures at blood levels less than or equal to 2 ng ml^{-1} (ppb), which is the approximate median and mean unconjugated circulating BPA level in humans. Therefore, the commonly reported circulating levels in humans exceed the circulating levels extrapolated from acute exposure studies in laboratory animals.
5. BPA levels in the fetal mouse exposed to BPA by maternal delivery of 25 μg kg^{-1}, a dose that has produced adverse effects in multiple experiments, are well within the range of unconjugated BPA levels observed in human fetal blood.

4.1.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Sensitivity to endocrine disruptors, including BPA, varies extensively with life stage, indicating that there are specific windows of increased sensitivity at multiple life stages. Therefore, it is essential to assess the impact of life stage on the response to BPA in studies involving wildlife, laboratory animals, and humans.
2. Developmental windows of susceptibility are comparable in vertebrate wildlife species and laboratory animals.
3. BPA alters "epigenetic programming" of genes in experimental animals and wildlife that results in persistent effects that are expressed later in life [25]. These organizational effects (functional and structural) in response to exposure to low doses of BPA during organogenesis persist into adulthood, long after the period of exposure has ended. Specifically, prenatal and/or neonatal exposure to low doses of BPA results in organizational changes in the prostate, breast, testis, mammary glands, body size, brain structure and chemistry, and behavior of laboratory animals.
4. There are effects due to exposure in adulthood that occurs at low doses of BPA. Substantial neurobehavioral effects and reproductive effects in both males and females have been observed during adult exposures in laboratory animals.
5. Adult exposure studies cannot be presumed to predict the results of exposure during development.
6. Life stage impacts the pharmacokinetics of BPA.

4.2. We believe the following to be likely but require confirmation

4.2.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. BPA metabolism occurs in cell culture systems, and although there are differences between cell types, there is less variability than in the entire animal. Metabolism is an important issue for humans and wildlife field populations with large genetic variability. Individual differences in BPA pharmacokinetics allow for underlying variability within a population, and may allow for the identification of sensitive and insensitive subpopulations.
2. The activity of various enzymes involved in drug, chemical, and hormone metabolism, as well as protection against oxidative stress, are programmed by hormone levels during sensitive periods in development. Developmental alterations in hormonal programming (activation or inhibition) may thus affect metabolism of BPA and other hormones and chemicals. Direct interaction of BPA with enzymes in cells has only been reported at higher doses than expected for human exposures.
3. The set of genes regulated by BPA is expected to differ among doses. Therefore, different doses of BPA do not produce different effects only due to a quantitative difference in the expression of the same set of genes.
4. Differential expression of estrogen receptor subtypes (α/ß; variant isoforms), and protein–protein interactions (estrogen receptor homo- and hetero-dimer formation, co-regulators, etc) modulate the cellular response to BPA. Direct actions of BPA on intracellular signal transduction modulate some cellular responses, which are similarly dependent on differential expression and protein–protein interactions.
5. Bioactive doses can be mathematically modeled, but further model refinement and experimental confirmation is required.
6. Other mechanisms (androgen receptors, thyroid hormone receptors) may be relevant for BPA action, but at higher doses than for estrogen responsive mechanisms.

4.2.2. Issue 2: Wildlife—laboratory animal research connection

1. The effects observed in laboratory animals could be present in wildlife, because the low doses being studied in laboratory animals are now relevant to environmental exposure levels of wildlife. The similarities in mechanisms that have been observed between different species suggest that field populations will respond to the same low levels.
2. Measurements of vitellogenin production in fish have established that there are exogenous estrogenic signals in the their environment. BPA may be contributing to this phenomenon as it enters natural water systems after leaching from landfills and due to plastic debris in water.
3. Delayed spawning is seen in male and female fish, which may relate to observed changes in estrous cyclicity in mammals in laboratory experiments.

4. In wildlife and laboratory studies, BPA induces alteration in steroid biosynthesis/ metabolism/excretion.
5. Wildlife residing in sediment is likely exposed to higher levels of BPA.

4.2.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure is likely to be continuous, unlike exposure in most laboratory animal studies of BPA pharmacokinetics.

4.2.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Clearance of BPA in the fetus is reduced compared to other life stages. Different effects and metabolic clearance mechanisms are also observed in neonatal and adult animals. Conjugation (glucuronidation) and other mechanisms of metabolic clearance of BPA thus vary throughout life.
2. Exposure to BPA during different life stages differentially influences reproductive cancer etiology and progression, and exposure during sensitive periods in organogenesis may increase susceptibility to development of cancers in some organs, such as the prostate and mammary glands.
3. Early life exposure to environmentally relevant BPA doses may result in persistent adverse effects in humans.
4. The function of the immune system can be altered following adult exposure to BPA.
5. Effects on insulin metabolism occur following adult exposure.

4.3. Areas of uncertainty and suggestions for future research

4.3.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. Since BPA can act as an agonist or an antagonist in different tissues and against different background physiological states, the specific co-regulators that mediated these different responses of BPA need to be elucidated based on *in vitro* mechanistic studies, which should be confirmed *in vivo*.
2. Research is needed on specific receptor sub-types (i.e., classical nuclear and non-classical membrane-associated estrogen receptors) in relation to the potency of BPA in different tissues.
3. The identification of multiple estrogen receptor genes and variants as well as different co-regulators with different activities reveals that different levels of potency of BPA could be obtained by complex interactions between these different components that would not be predicted in homogeneous recombinant systems.

4.3.2. Issue 2: Wildlife—laboratory animal research connection

1. To directly relate the effects seen in wildlife with BPA exposure, biomonitoring data are needed from wildlife. In addition to BPA levels, these studies should assay total estrogenic and antiandrogenic activity from other contaminants.
2. There is a need to examine sensitive endpoints in wildlife that have been identified in laboratory animals.
3. There are substantial amounts of plastic debris within marine and fresh water ecosystems, and studies are needed to examine the impact of BPA in the environment on aquatic organisms. Doses used in laboratory experiments involving wildlife should reflect environmental exposures.
4. More studies need to be done with BPA in invertebrates, and a fundamental understanding of estrogen action in invertebrates is required.
5. Studies should determine if amplification of BPA through the food chain occurs, particularly under anaerobic or hypoxic conditions due to the lack of microbial or photodegradation.
6. Future research emphasis should be placed on populations of aquatic animals exposed to landfill leachate and sewage effluent, as these are the primary point sources for BPA exposure.

4.3.3. Issue 3: Laboratory animal research—human exposure connection

1. Even though there have been attempts to estimate daily human intake of BPA, these estimates require many assumptions. The best measures we have to estimate whether humans may be affected by current exposures to BPA are levels in blood (not exposure levels), which can be related to blood levels in experimental animals after acute exposures. Known sources of human exposure to BPA do not appear sufficient to explain levels measured in human tissues and fluids.
2. While BPA is not persistent in the environment or in humans, biomonitoring surveys indicate that exposure is continuous. This is problematic because acute animal exposure studies are used to estimate daily human exposure to BPA, and at this time, we are not aware of any studies that have examined BPA pharmacokinetics in animal models following continuous low level exposures. Measurement of BPA levels in serum and other body fluids suggests that either BPA intake is much higher than accounted for, or that BPA can bioaccumulate in some conditions such as pregnancy, or both. Research using both animal models, as well as epidemiology studies, are needed to address these hypotheses, and this research needs to better mimic the apparent continuous exposure of humans to BPA.
3. More comprehensive exposure and biomonitoring studies are needed, especially in developing countries.
4. In both animal and human studies, internal exposure measures need to be related to health effects. In particular, there is a need for epidemiological studies relating health outcomes to BPA exposure, particularly during sensitive periods in development. These studies should be based on hypotheses from findings in experimental animals. This will require additional development of appropriate biomarkers in animal studies that can be used in epidemiological research.

4.3.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. While there is a great need to continue studying prenatal and perinatal exposures in laboratory animal studies, many organs and endpoints continue developing at later stages (throughout puberty and adolescence). Additional studies are needed during these later periods of development.
2. Additional research is needed regarding exposure to BPA in adulthood to determine whether post-exposure effects are temporary or are permanent and associated with subsequent age-related diseases.
3. Because aging adults lose repair mechanisms, metabolic enzymes, and imprinted genes, the possibility that adult exposures (long-term, low level) can increase the risk of cancers and other conditions during aging should be addressed with additional human research and the development of appropriate animal models.
4. Epigenetics should be examined as a potential mechanism mediating developmental effects as well as the trans-generational effects of BPA and other contaminants. Potential effects of adult exposures also need to be examined in relation to disruption of epigenetic changes that occur normally during aging.
5. Trans- and multi-generational effects of BPA must be examined in laboratory animals and humans.
6. There is a need for studies that involve collection of human blood and urine from humans at several life stages, with specific emphasis on infants and young children and continued monitoring throughout adulthood. Additionally, there is a need to characterize the basis for the variability in BPA levels in studies examining both human urine and serum.
7. There is a need for research on the genetic basis for differences in susceptibility to BPA and other contaminants.
8. Studies are needed on comparative BPA pharmacokinetics in invertebrates and vertebrates (non-human primates included).
9. There is a need to measure total endocrine disrupter load in humans and wildlife. Therefore, biomarkers of endocrine disrupter exposure are necessary.
10. There is a need for more research directed at examining human exposure, pharmacokinetics and health effects of selected BPA precursors (i.e., BADGE, BISGMA, and BISDMA) and metabolites (e.g., halogenated BPAs).
11. There is a need for more studies focused on identification of other (non-estrogen-receptor mediated) mechanisms of action of BPA.
12. Effects of chemicals on the immune system are life stage dependent, and identifying the life stage dependency for BPA effects on the immune system is necessary. In addition, studies examining BPA effects on the immune system in wildlife are necessary.

5. Conclusions

The published scientific literature on human and animal exposure to low doses of BPA in relation to *in vitro* mechanistic studies reveals that human exposure to BPA is within the range that is predicted to be biologically active in over 95% of people sampled. The wide range of adverse effects of low doses of BPA in laboratory animals exposed both during development and in adulthood is a great cause for concern with regard to the potential for similar adverse effects in humans. Recent trends in human diseases relate to adverse effects observed in experimental animals exposed to low doses of BPA. Specific examples include: the increase in prostate and breast cancer, uro-genital abnormalities in male babies, a decline in semen quality in men, early onset of puberty in girls, metabolic disorders including insulin resistant (type 2) diabetes and obesity, and neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD).

There is extensive evidence that outcomes may not become apparent until long after BPA exposure during development has occurred. The issue of a very long latency for effects *in utero* to be observed is referred to as the developmental origins of adult health and disease (DOHaD) hypothesis. These developmental effects are irreversible and can occur due to low dose exposure during brief sensitive periods in development, even though no BPA may be detected when the damage or disease is expressed. However, this does not diminish our concern for adult exposure, where many adverse outcomes are observed while exposure is occurring. Concern regarding exposure throughout life is based on evidence that there is chronic, low level exposure of virtually everyone in developed countries to BPA. These findings indicate that acute studies in animals, particularly traditional toxicological studies that only involve the use of high doses of BPA, do not reflect the situation in humans.

The fact that very few epidemiological studies have been conducted to address the issue of the potential for BPA to impact human health is a concern, and more research is clearly needed. This also applies to wildlife, both aquatic and terrestrial. The formulation of hypotheses for the epidemiological and ecological studies can be greatly facilitated by the extensive evidence from laboratory animal studies, particularly when common mechanisms that could plausibly mediate the responses are known to be very similar in the laboratory animal models, wildlife and humans.

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The negative regulation of Mesp2 by mouse Ripply2 is required to establish the rostro-caudal patterning within a somite

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The Mesp2 transcription factor plays essential roles in segmental border formation and in the establishment of rostro-caudal patterning within a somite. A possible Mesp2 target gene, *Ripply2*, was identified by microarray as being downregulated in the *Mesp2*-null mouse. *Ripply2* encodes a putative transcriptional co-repressor containing a WRPW motif. We find that Mesp2 binds to the *Ripply2* gene enhancer, indicating that *Ripply2* is a direct target of Mesp2. We then examined whether Ripply2 is responsible for the repression of genes under the control of Mesp2 by generating a *Ripply2*-knockout mouse. Unexpectedly, *Ripply2*-null embryos show a rostralized phenotype, in contrast to *Mesp2*-null mice. Gene expression studies together with genetic analyses further revealed that Ripply2 is a negative regulator of Mesp2 and that the loss of the *Ripply2* gene results in the prolonged expression of Mesp2, leading to a rostralized phenotype via the suppression of Notch signaling. Our study demonstrates that a Ripply2-Mesp2 negative-feedback loop is essential for the periodic generation of the rostro-caudal polarity within a somite.

KEY WORDS: Somitogenesis, Notch signaling, Presomitic mesoderm, Segmentation

INTRODUCTION

Somites are generated by sequential segregation of cell masses from the anterior part of the unsegmented presomitic mesoderm (PSM), in both a spatially and temporally coordinated manner every two hours (Iulianella et al., 2003; Pourquie, 2003; Saga and Takeda, 2001). The somites provide the basic axial structures that underlie the segmental architecture of not only the vertebra, ribs and muscles, which are all somite derivatives, but also of the vascular and nervous systems (Borycki and Emerson, 2000; Brand-Saberi and Christ, 2000; Monsoro-Burq and Le Douarin, 2000). Periodicity is generated by Notch signal oscillations linked to the segmentation clock (Bessho et al., 2001; Huppert et al., 2005; Morimoto et al., 2005; Rida et al., 2004). The temporal information that results from this is translated into spatial patterns in the anterior PSM, which is defined by the so-called determination front (Dubrulle and Pourquie, 2004).

The Mesp2 transcription factor plays important roles during somitogenesis (Saga et al., 1997), and its expression is periodically activated by cyclic Notch signaling and Tbx6 at the anterior PSM in the determination front (Yasuhiko et al., 2006). Mesp2 demarcates the next segmental boundary and defines the rostro-caudal identity of somites (Takahashi et al., 2000). It has been shown that *Mesp2*-null embryos fail to segment and that the resulting non-segmented somites show caudalized properties (Saga et al., 1997). Previously, we have shown that Mesp2 suppresses Notch activity via the activation of *Lfng*, which might function as a negative regulator of Notch signaling (Morimoto et al., 2005). In addition, Mesp2 acts as the transcriptional activator of *Epha4* in the anterior PSM (Nakajima et al., 2006). Mesp2 is also known to be a strong suppressor of genes such as *Dll1* and *Uncx4.1* that confer caudal properties upon the somitic cells via Notch signaling (Takahashi et al., 2000). However, the manner in which the caudal genes are suppressed is currently unknown. In our current study, which aimed to elucidate the molecular mechanisms underlying the regulation of somitogenesis by Mesp2, we have compared the gene expression patterns of $Mesp2^{+/-}$ and $Mesp2^{-/-}$ embryos, and found that several genes are affected by the *Mesp2* knockout. Among the downregulated genes that we identified in the *Mesp2*-null embryo, we focused on a putative transcriptional repressor. This gene turned out to be *Ripply2*, which was recently reported as a mouse homolog of zebrafish *ripply1* (Kawamura et al., 2005). Morpholino-mediated knockdown analysis revealed that *ripply1* is required for the proper transition from the PSM to somites. We generated a *Ripply2*-knockout mouse and now show that *Ripply2* is activated by Mesp2, but also functions negatively toward Mesp2 to regulate the levels of Notch signaling in the anterior PSM. This negative regulation is required for the periodic generation of the rostro-caudal patterning within a somite.

MATERIALS AND METHODS

GeneChip analysis

Total RNA was purified from cells corresponding to the S–1 to S2 somites and PSM of wild-type, *Mesp2-GFP* knock-in heterozygous and homozygous embryos at E10.5 using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNAs were synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen) and 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′]. After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip Sample Cleanup Module (Affymetrix), according to the manufacturer's instructions. Our detailed methods for the labeling of the

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double-stranded cDNAs and hybridization to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix), and the subsequent washing, staining and data analysis have been described previously (Kanno et al., 2006). All of these data are also now available online at the National Institute of Health Sciences (http://www.nihs.go.jp/tox/TtgSubmitted.htm).

Identification of the *Ripply2* somite enhancer

Highly conserved *Ripply2* upstream regions were identified using a cross-species DNA sequence comparison using the PipMaker website (http://pipmaker.bx.psu.edu/pipmaker/). We cloned a 5′ upstream genomic sequence of *Ripply2* from a bacterial artificial clone (RP23) contained in a mouse genomic library. A 1.5 kb DNA fragment containing the 171 bp highly conserved region was isolated by *Eco*RI and *Bam*HI digestion and then subcloned into the hsp-nlacZ reporter construct. Fertilized eggs from B6C3F1 female mice were collected for pronuclear injection and the injected eggs were then implanted into ICR female mice. Foster mothers were sacrificed at E10.5 and stained for β-galactosidase (β-gal) activity with X-Gal. The genotypes of the embryos were then identified by PCR using DNA prepared from the yolk sac.

Luciferase assay

For luciferase reporter analysis under the control of the 1.5 kb *Ripply2* anterior-PSM enhancer (*Eco*RI-*Bam*HI) fragment (20 ng), reporter constructs were co-transfected with the expression vectors 3xFLAG-Mesp2 (0, 30, 100 ng) and 3xFLAG-E47 (0, 50 ng) into NIH3T3 cells (0.25×10^5 cells per well in 24-multiwell plates) using Lipofectamine Plus (Invitrogen), following the manufacturer's instructions. The vector containing the *Renilla* luciferase gene under the control of the thymidine kinase promoter (1 ng) was co-transfected as an internal standard to normalize for transfection efficiency, and the amount of total plasmid was adjusted with pcDNA3.1. After 36 hours, luciferase activities were measured using a Dual Luciferase Assay Kit (Promega).

Electrophoretic mobility shift assay (EMSA)

A 3xFLAG-Mesp2 protein was produced using the FreeStyle 293 Expression System (Invitrogen) and then collected via a nuclear extraction method. Double-stranded DNA oligonucleotide probes were end-labeled with DIG and protein-DNA complexes were detected using a DIG Gel Shift Kit (Roche). Binding reactions were carried out for 30 minutes on ice, and protein-DNA complexes were analyzed on 6% native polyacrylamide gels.

Ripply2 gene targeting strategy

The mouse *Ripply2* gene consists of four exons, the first of which harbors two putative in-frame translational initiation codons. We generated a targeting vector with a floxed *neo* cassette to remove a portion of exon 1, which would introduce a termination codon just after the second initiation codon and produce a null allele. The resulting linearized vector (25 μg) was then electroporated into TT2 ES cells (Yagi et al., 1993). G418-resistant cell clones were further selected by PCR. Correct homologous recombination was confirmed by Southern blotting, and targeted cell clones were aggregated with ICR 8-cells and then transferred to pseudopregnant female recipients. The resulting chimeric mice were bred with ICR females. Germline transmission of the targeted allele was confirmed by PCR. The floxed neomycin cassette was later removed by breeding with a CAG-Cre transgenic mouse (Sakai and Miyazaki, 1997).

Gene expression and histochemical analysis

Methods for gene expression analysis by in situ hybridization of whole-mount samples and by skeletal staining have been described previously (Takahashi et al., 2000). The probes used in this study have been described previously (Takahashi et al., 2000; Takahashi et al., 2003; Nomura-Kitabayashi et al., 2002). For the *Ripply2* RNA probe, we used a full-length cDNA clone containing intron 1. Section in situ hybridization and immunohistochemical detection of proteins were performed as previously described (Morimoto et al., 2005). For whole-mount detection of Mesp2-venus, embryos were fixed with 4% paraformaldehyde in PBS overnight at 4°C, incubated with rabbit anti-GFP (MBL; 1:1000), followed by Alexa-488-conjugated goat anti-rabbit IgG (Molecular Probes; 1:400) and observed using a fluorescent microscope (Olympus BX61).

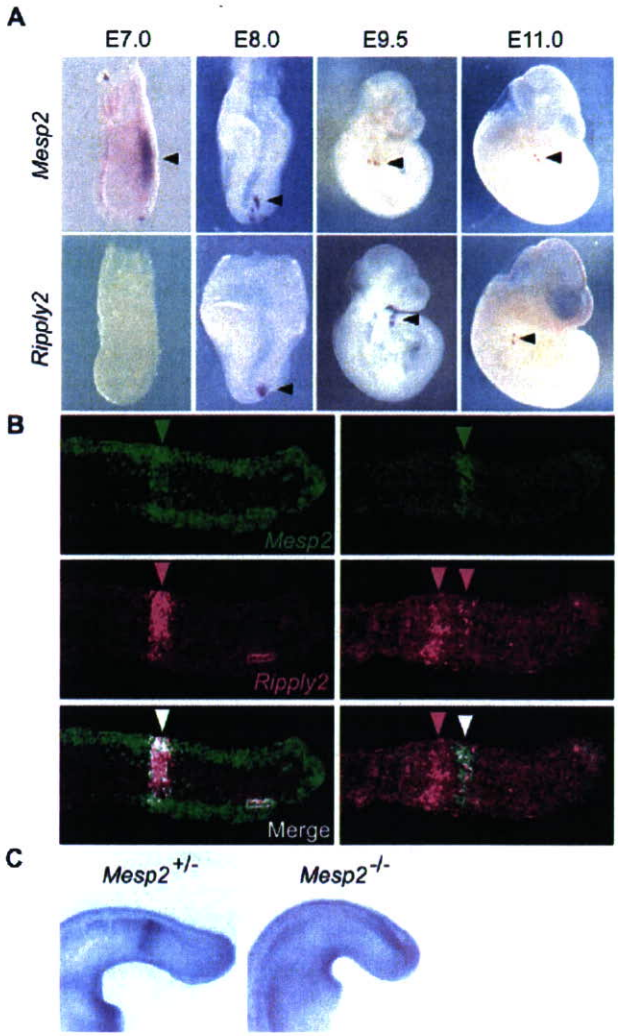

Fig. 1. Analysis of the of *Ripply2* expression pattern. (**A**) Comparison of the mRNA expression patterns of *Mesp2* and *Ripply2* during mouse development. Positive expression is indicated by an arrowhead. (**B**) Comparison of the spatial expression patterns of *Mesp2* and *Ripply2* as revealed by section double in situ hybridization. Two representative examples are shown for *Mesp2* (green) and *Ripply2* (magenta), and merged images of these expression patterns are shown beneath. The green signals in the periphery are artifacts and do not represent *Mesp2* expression. In some cases, only a single band could be observed for each gene, and these bands are merged in the image shown in the left-hand bottom panel. Two bands were sometimes visible for *Ripply2*, the posterior band of which merges with that of *Mesp2* (right-hand bottom panel). All samples were prepared from E10.5 embryos. (**C**) Whole-mount in situ hybridization showing that *Ripply2* expression is lost in the E9.5 *Mesp2*-null embryo.

RESULTS

Ripply2 is a possible direct target of Mesp2

Mesp2 is known to function as a transcriptional activator of genes, such as *Epha4* and *Lfng*, which are expressed in the rostral half of the presumptive somite (Morimoto et al., 2005; Nakajima et al., 2006). Moreover, expression of the *Dll1* and *Uncx4.1* genes, which are expressed in the caudal half of the somite (Bettenhausen et al.,

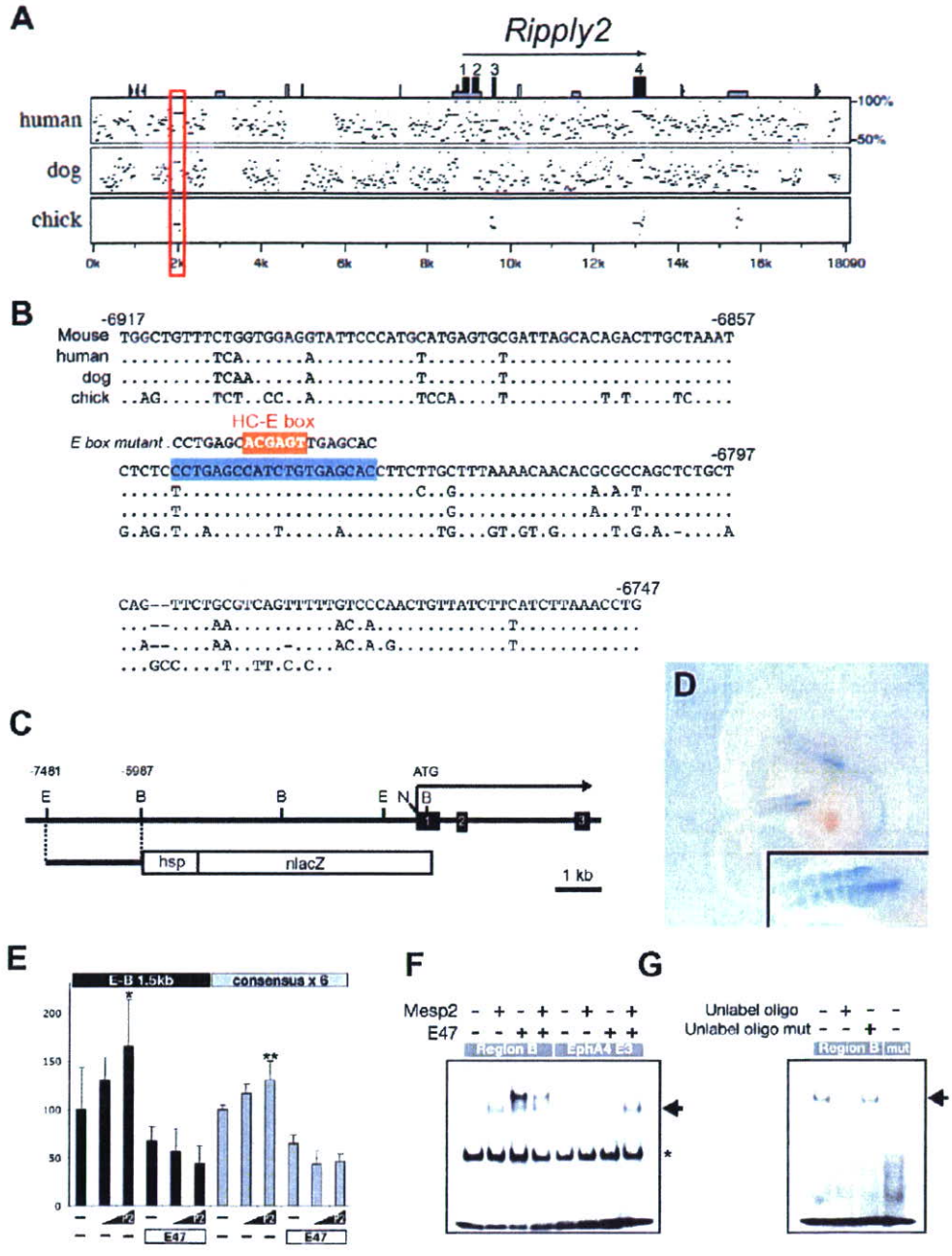

Fig. 2. Mesp2 can directly bind to the enhancer element of the *Ripply2* gene and activate its transcription. (**A**) Comparison of the genomic sequences around the *Ripply2* gene in mouse (top line) with those in human, dog and chick using MultiPipMaker sequence alignment software. A conserved region (framed in red) is evident across these species. (**B**) Sequence alignment of the 171 bp region conserved among the *Ripply2* genes, within which a highly conserved E-box is located. HC E-box, highly conserved E-box. (**C**) The genomic organization of the mouse *Ripply2* gene and the corresponding construct used in the transgenic analyses. A 1.5 kb DNA fragment containing this highly conserved 171 bp stretch (shown in A) of the *Ripply2* upstream region was ligated to a cassette composed of the *hsp* promoter and nlacZ (*lacZ* harboring a nuclear localization signal). E, *Eco*RI; B, *Bam*HI; N, *Nco*I. (**D**) The *Ripply2* enhancer drives *lacZ* reporter gene expression in somitic mesoderm cells at E11.0. The inset shows high magnification of the somitic region. (**E**) Luciferase reporter assay for *Mesp2* activation, with or without E47, using constructs harboring either the 1.5 kb *Ripply2* enhancer (left) or six repeats of the conserved 171 bp fragment (right). The addition of E47 had negative effects upon transactivation. The data represent the means±s.d. from four separate experiments. $*P<0.01$, $**P<0.04$. (**F**) EMSA analyses revealing that a DNA fragment containing the conserved E-box (Region B, light-blue shading) from the *Ripply2* upstream region can bind Mesp2 in the absence of E47. This binding of Mesp2 thus appears to be different from its binding to the *Epha4* enhancer, which is dependant upon E47. Non-specific bands are indicated by the asterisk. (**G**) The binding specificity of Mesp2 was confirmed by successful competition with cold probe, but not with an E-box mutant probe (shown in B).

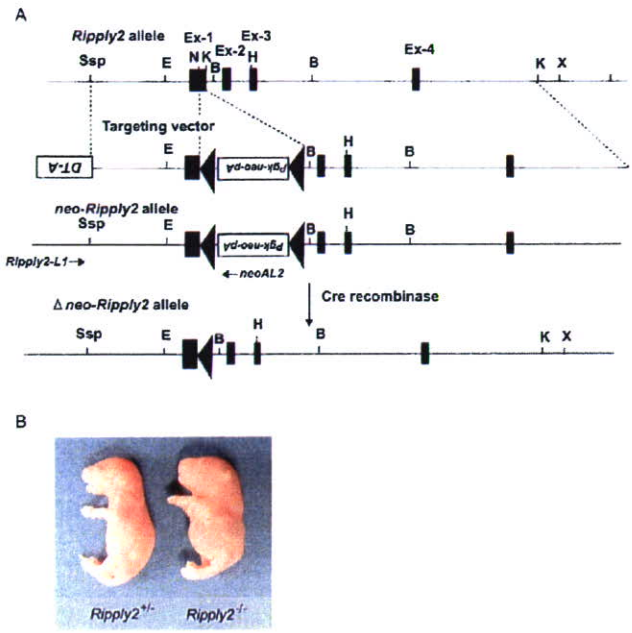

Fig. 3. The targeting strategy used for the *Ripply2* gene and the external morphology of the resulting knockout mouse. (**A**) The top line shows the genomic organization of the *Ripply2* gene, the second line represents the structure of the targeting vector, and the bottom two lines show the predicted structure of the *Ripply2* locus following homologous recombination. The first exon of *Ripply2* was partially deleted and replaced with a floxed *neo* cassette (the arrowheads on the line represent loxP sites). A germline chimeric mouse was then generated from recombinant ES cells containing the targeted allele and crossed with a CAG-Cre mouse to remove the *neo* cassette and establish the *Ripply2*-knockout mouse line. Ssp, *Ssp*I; E, *Eco*RI; B, *Bam*HI; H, *Hind*III; N, *Nco*I; K, *Kpn*I; X, *Xho*I. (**B**) The *Ripply2*-null mouse dies soon after birth and the external morphology at E17.5 is similar to those of segmentation-defective mutants, featuring a short trunk with rudimental tails.

1995; Leitges et al., 2000), is increased in the *Mesp2*-null mouse, indicating that Mesp2 is required for their suppression (Takahashi et al., 2000). However, the molecular mechanisms underlying this are unknown. To identify novel genes that operate downstream of Mesp2, we performed GeneChip analysis using RNAs prepared from both wild-type and *Mesp2*-null embryos. Among the genes that showed a reduction in expression in the *Mesp2*-null embryos (see Table S1 in the supplementary material), we selected a cDNA clone (corresponding to RIKEN cDNA C030002E08) that showed an identical expression pattern to that of *Mesp2* by in situ screening of 11.5 dpc embryos. This cDNA was subsequently revealed to be the mouse *Ripply2* gene recently reported by Kawamura et al. (Kawamura et al., 2005). The initial expression of *Mesp2* was found to be restricted to the nascent mesoderm at E7.0, but *Ripply2* expression appeared to be absent or very weak prior to somitogenesis (Fig. 1A). However, its expression became evident in the anterior PSM as a pair of bands by 8.0 dpc, similar to *Mesp2*

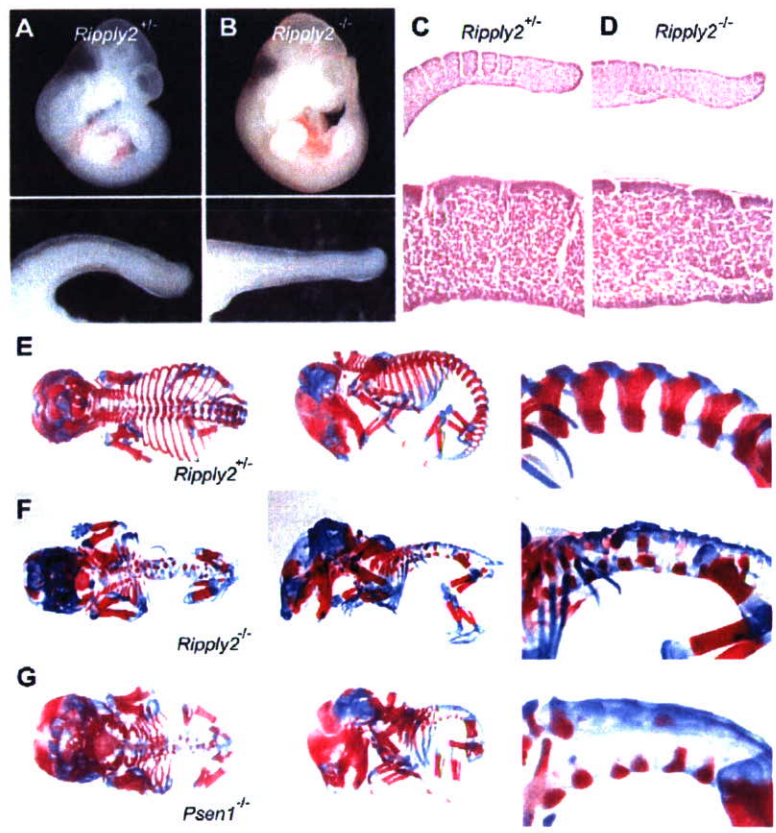

Fig. 4. The *Ripply2*-knockout mouse exhibits segmentation defects. (**A-D**) *Ripply2*$^{+/-}$ and *Ripply2*$^{-/-}$ embryos (n=3 at E10.5) were compared by external morphology (A,B) and by the Hematoxylin and Eosin staining of parasagittal sections of tail regions (C,D). *Ripply2*$^{-/-}$ embryos display irregularly sized myotomes, and an unclear segmental border. (**E-G**) Skeletal preparations at E17.5 stained with Alizarin Red-Alcian Blue reveal that the *Ripply2*$^{-/-}$ fetus harbors fewer pedicles of neural arches and lacks components of the proximal ribs (F; n=4), which is similar to the aberrant phenotype of the *Psen1*-null fetus (G; n=2).

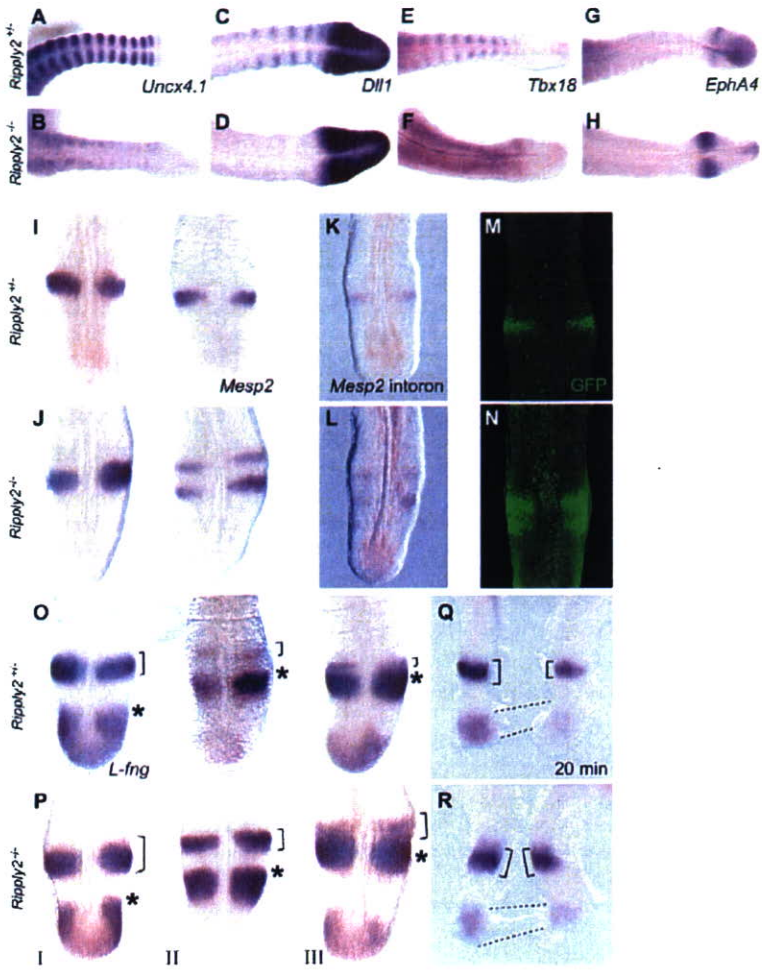

Fig. 5. Altered gene expression in the *Ripply2*-null embryos. Whole-mount in situ hybridizations were employed to characterize somitogenesis in the *Ripply2*$^{-/-}$ embryo. The expression of caudal genes such as *Uncx4.1* (**A**,**B**) and *Dll1* (**C**,**D**) was found to be reduced, whereas rostral genes such as *Tbx18* (**E**,**F**) and *Epha4* (**G**,**H**) show an expanded pattern in *Ripply2*$^{-/-}$ embryos at E11.5. (**I-N**) Comparisons of the expression patterns of *Mesp2* mRNA, detected by exon (I,J) and intron (K,L) probes, and protein levels (M,N), at E10.5. An additional *Mesp2* expression band appears rostrally in the *Ripply2*$^{-/-}$ embryos (J,L). Mesp2 protein expression, visualized by Mesp2-venus, was compared between the *Ripply2*$^{+/-}$ (M, *n*=2) and *Ripply2*$^{-/-}$ (N, *n*=3) genetic backgrounds. The confocal images were visualized by fluorescence, detected using anti-GFP antibodies. (**O**,**P**) Comparison of the *Lfng* expression patterns at different cyclic phases (indicated by I to III) at E10.5. The oscillatory expression of *Lfng* (asterisks) in the posterior PSM was unaffected, but the rostral-most expression bands (brackets) are slightly expanded in the *Ripply2*$^{-/-}$ embryos (P), as compared with the *Ripply2*$^{+/-}$ embryos (O). (**Q**,**R**) The prolonged expression of *Lfng* in the anterior PSM. The PSM of E10.5 *Ripply2*$^{+/-}$ (Q) and *Ripply2*$^{-/-}$ (R) embryos was separated into two halves, with one being fixed immediately and the other fixed after explant culturing for 20 minutes. Both were then analyzed for *Lfng* mRNA. The expression of *Lfng* in the anterior PSM is maintained for longer in the *Ripply2*$^{-/-}$ embryos.

(Fig. 1A). The expression of *Ripply2* then continued until 12.5 dpc, during the somite-forming period (Fig. 1A and data not shown). The expression domains of *Mesp2* and *Ripply2* were next compared by double in situ hybridization of embryonic tail sections. Two typical patterns are shown in Fig. 1B. One shows single bands that are completely merged, whereas the other is of a single *Mesp2* band and two *Ripply2* bands in which the caudal band is merged with a distinct *Mesp2* band. This observation indicates that *Mesp2* expression precedes that of *Ripply2*, but that *Ripply2* persists for longer. In addition, *Ripply2* expression was lost in the *Mesp2*-null embryo (Fig. 1C), as predicted from our GeneChip analysis. These data thus indicated that *Ripply2* might be a target of Mesp2.

To examine this possibility, we searched for possible cis-regulatory sequences in the *Ripply2* gene by comparing mouse, human, dog and chick genomic sequences using MultiPipMaker sequence alignment software (Fig. 2A). From these analyses, we identified a conserved region (–6917 to –6747, Fig. 2B). To investigate whether the 1.5 kb region containing this conserved 171 bp sequence (Fig. 2B) possessed enhancer activity, we performed transient transgenic analyses using a β-gal reporter (Fig. 2C). In five out of nine PCR-positive embryos, we detected specific β-gal expression in several segmented somites (Fig. 2D), which is a typical pattern for genes expressed in the anterior PSM, including *Mesp2* and *Epha4* (Haraguchi et al., 2001; Nakajima et al., 2006). We next employed a luciferase reporter assay system to ascertain whether the enhancer activity was dependant upon Mesp2. Two reporter constructs were generated – one containing the 1.5 kb genomic fragment and the other harboring six repeats of the 171 bp consensus sequence. Both constructs were activated by the addition of Mesp2, but not in conjunction with E47 (also known as Tcfe2a – Mouse Genome Informatics) (Fig. 2E). This result was different from the findings of our previous study of the *Epha4* enhancer (Nakajima et al., 2006), in which Mesp2 was observed to bind and transactivate the reporter activity only in the presence of E47, a possible heterodimeric partner. Since Mesp2 belongs to the bHLH-type transcription factor family, which is known to bind either to E-box or N-box motifs, we screened the 171 bp *Ripply2* gene consensus sequence for E-boxes, or for an N-box which is capable of binding to Mesp2 with or without E47. We identified a DNA fragment containing a highly conserved E-box CATCTG sequence, and confirmed that this binds to Mesp2, whereas a mutated form did not (Fig. 2F,G). E47 was also found to bind to this E-box, but this might not be functional binding as no associated activity was detectable by luciferase reporter assay. Furthermore, the binding of Mesp2 was weakened by the addition of E47. These results are consistent with the idea that Mesp2 binds to this E-box in the enhancer of the *Ripply2* gene, and that this enhancer does not require E47 for subsequent transactivation.

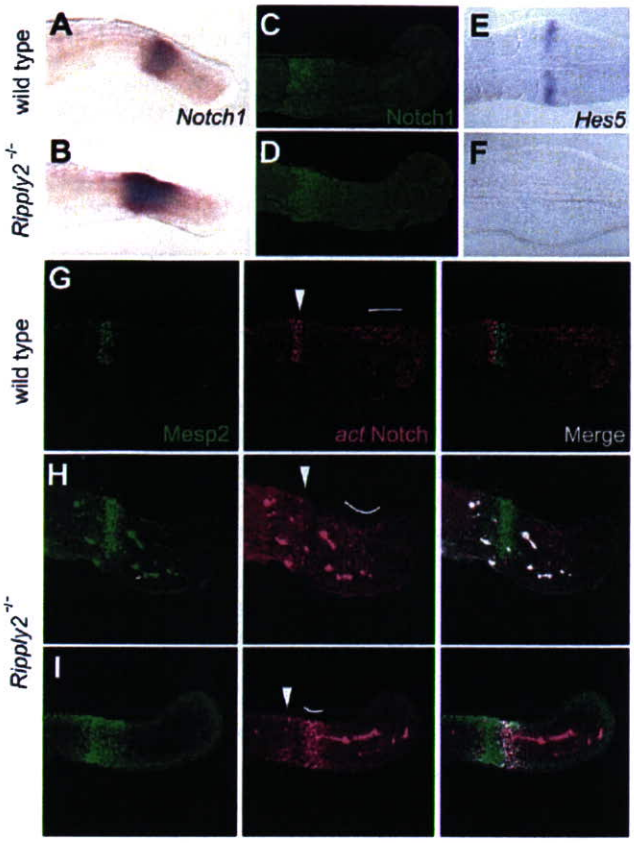

Fig. 6. Notch signaling is reduced in the anterior PSM in the *Ripply2*$^{-/-}$ embryo. (**A-F**) *Notch1* mRNA (A, *n*=2; B, *n*=2), Notch1 protein (C, *n*=2; D, *n*=2) and *Hes5* mRNA (E, *n*=2; F, *n*=4) expression patterns were compared between wild-type (A,C,E) and *Ripply2*$^{-/-}$ (B,D,F) embryos at E11.0. (**G-I**) Double immunostaining with anti-Mesp2 (green) and anti-active Notch1 (magenta; the white lines indicate activities in the anterior PSM) antibodies using sections of wild-type (G) and *Ripply2*$^{-/-}$ (H,I) E11.0 embryos. In the *Ripply2*$^{-/-}$ background, Mesp2 expression is upregulated but Notch activity is reduced.

The *Ripply2*-knockout mouse exhibits a rostralized phenotype

Because Mesp2 confers rostral properties to the somites and is involved in the formation of the somite boundary, we speculated whether Ripply2 might function in this Mesp2 pathway during somitogenesis. To elucidate this possibility, we generated *Ripply2*-knockout mice using ES cell-mediated gene targeting (Fig. 3A). Since the heterozygous mice were found to be normal, we performed timed intercross matings to analyze the phenotypes of the homozygotes. As expected from the expression patterns, the *Ripply2*$^{-/-}$ embryos failed to proceed through normal somitogenesis and the embryos displayed no clear segmental borders (Fig. 4A-D). These homozygous mice also died soon after birth. The morphology of the 17.5-dpc fetus was found to be similar to that of the *Mesp2*-null embryo, with a short trunk and tail (Fig. 3B) (Saga et al., 1997). However, the vertebral phenotype of the *Ripply2*$^{-/-}$ embryos, as revealed by skeletal staining, differed from that of *Mesp2*-null embryos as it features extensive fusion of the pedicles in the neural arches owing to the caudalized characteristics of the somitic mesoderm (Saga et al., 1997).

The *Ripply2*$^{-/-}$ mouse fetus showed fewer pedicles of neural arches (Fig. 4E,F), and the phenotype resembled that of the presenilin 1 (*Psen1*)-null mouse (Fig. 4G), which lacks Notch signaling (Koizumi et al., 2001). The findings of our gene expression studies using both rostral and caudal molecular markers are consistent with these skeletal defects. In *Ripply2*$^{+/-}$ embryos, the expression of the caudal markers *Uncx4.1* and *Dll1* in the segmented somites was restricted to the caudal compartments of the somites (Fig. 5A,C). The expression of these genes is increased and more expansive in *Mesp2*-null embryos (Takahashi et al., 2000), but was greatly reduced in the *Ripply2*-null embryos, (Fig. 5B,D). In addition, no *Dll1* stripe could be observed within the somitic region or in the anterior PSM, although the expression in the posterior PSM was intact in the *Ripply2*$^{-/-}$ embryo (Fig. 5D). By contrast, the rostral markers were found to be present in the *Ripply2*$^{-/-}$ embryo (Fig. 5E-H). *Tbx18*, which is known to be involved in the maintenance of the rostral properties of the somites (Bussen et al., 2004; Kraus et al., 2001), was expressed in the rostral compartment of the segmented somites (Fig. 5E). In *Ripply2*$^{-/-}$ embryos, this expression was expanded throughout the entire somite region and no clear segmental pattern was evident (Fig. 5F). In addition, *Epha4* was expressed in the rostral compartment of S0 and S1 somites in the *Ripply2*$^{+/-}$ embryo (Fig. 5G), and this expression in the *Ripply2*$^{-/-}$ embryo was increased and the expression domain expanded as compared with the wild type (Fig. 5H). We thus conclude that the *Ripply2*-null mouse displays a rostralized phenotype.

Ripply2 is a negative regulator of *Mesp2* expression

As we have previously reported, the rostro-caudal polarity of the somites is generated by the interaction between Mesp2 and the Notch signaling pathway in the anterior PSM (Morimoto et al., 2005). To identify the underlying cause of the rostralized phenotype in the *Ripply2*-null embryo, the *Mesp2* gene expression profile was examined. During somitogenesis in the anterior PSM, both wild-type and *Ripply2*$^{+/-}$ embryos generally showed either a single *Mesp2* expression band of variable width or no band, depending on the cyclic expression stage (Fig. 5I and data not shown). However, we observed that *Mesp2* is expressed in the *Ripply2*$^{-/-}$ embryo as either one or two bands (Fig. 5J). In other words, an additional band was frequently observed in the more-rostral region (four out of six examined). In addition, we did not observe any *Ripply2*$^{-/-}$ embryos without *Mesp2* expression, suggesting either that *Mesp2* expression is prolonged or that the *Mesp2* transcripts are stabilized in a *Ripply2*$^{-/-}$ background. To distinguish these possibilities, we performed in situ hybridization using an intron probe. Although the signal obtained was low, we frequently detected two bands in the *Ripply2*$^{-/-}$ embryos (Fig. 5L; in all three examined), but only one band in the *Ripply2*$^{+/-}$ embryos (Fig. 5K). Hence, the transcription of *Mesp2* appears to be prolonged in the absence of Ripply2, although the possibility that differences exist in their mRNA stability cannot yet be excluded.

We next examined how the expression of the Mesp2 protein is influenced in the *Ripply2*$^{-/-}$ background. As we have shown previously, Mesp2-venus can be used to visualize functional Mesp2 proteins in vivo because the homozygous knock-in mouse is viable and shows normal somitogenesis (Morimoto et al., 2005). In a typical case, a single Mesp2-venus band was detectable in the area just caudal to the next presumptive segmental border in the *Ripply2*$^{+/-}$ background (Fig. 5M). However, in the *Ripply2*$^{-/-}$ embryo, two broader and interconnected bands could be discerned (Fig. 5N). These data

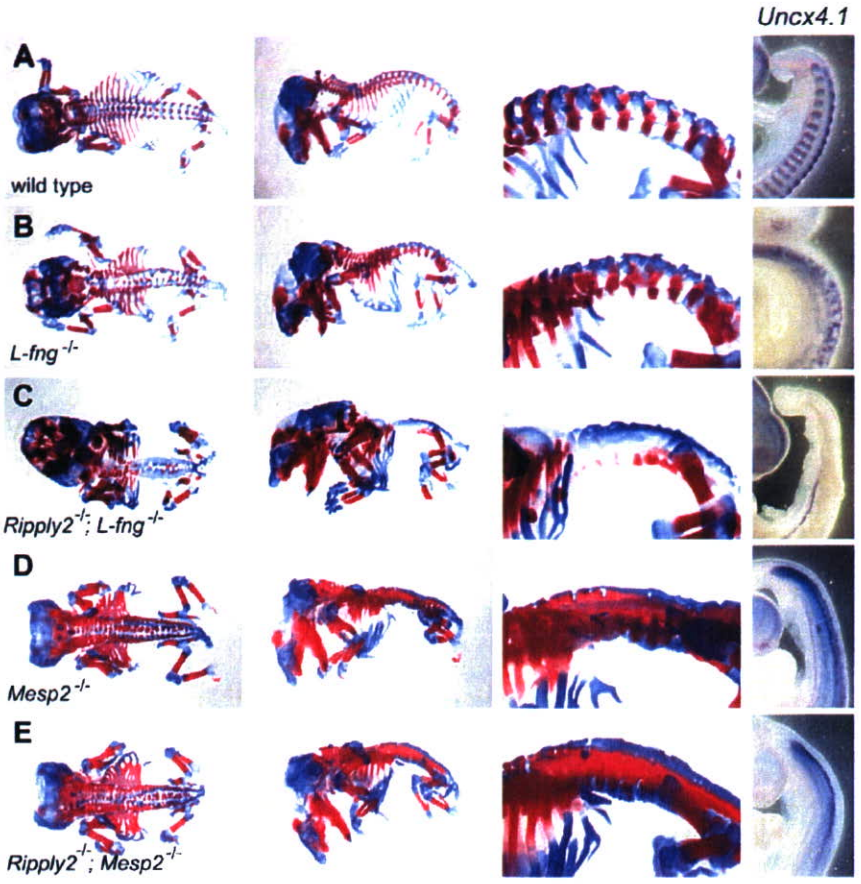

Fig. 7. Genetic analyses using double-knockouts of *Ripply2* and either *Lfng* or *Mesp2*. The skeletal morphologies and *Uncx4.1* expression patterns were compared among wild-type (**A**), *Lfng*-null (**B**), *Ripply2/Lfng* double-null (**C**), *Mesp2*-null (**D**) and *Ripply2/Mesp2* double-null (**E**) E17.5 fetuses or E9.5 embryos. The skeletal defects in the *Ripply2*$^{-/-}$ fetus were found to be further enhanced by the additional loss of *Lfng*, and the pedicles of the neural arches were almost completely absent in this compound-null fetus (C). By contrast, the *Ripply2/Mesp2* double-null fetus (E) shows a similar morphology to that of the *Mesp2* single-null fetus (D). The *Uncx4.1* expression pattern was independently examined at E10.5 (A, n=2; B, n=2; C, n=1) and E9.5 (A, n=4; B, n=2; C, n=2; D, n=4; E, n=2). Only representative images of E9.5 embryos are shown.

suggest that *Mesp2* is negatively regulated by Ripply2, and that these factors form a negative-feedback loop to restrict the levels of Mesp2.

We previously reported that *Lfng* expression is activated by Mesp2 in the anterior PSM and is subsequently involved in the suppression of Notch signaling. Moreover, *Lfng* expression shows a cyclic wave-like pattern in the posterior PSM, but its expression in the anterior PSM is similar to that of *Mesp2* in *Ripply2*$^{+/-}$ embryos. The width of this *Lfng* band becomes thinner before disappearing from the rostral end of the expression domain in the *Ripply2*$^{+/-}$ embryo (Fig. 5O). However, in the *Ripply2*$^{-/-}$ embryos, the anterior-most *Lfng* band was found to be wider and to persist for much longer as compared with the *Ripply2*$^{+/-}$ embryos (Fig. 5P). This persistent expression of *Lfng* was also evident from 20-minute explant culture experiments with a half-PSM (Fig. 5Q,R). These results suggest that Notch signaling might be suppressed, even in the presumptive caudal compartment of the somites, by prolonged Mesp2 and/or Lfng expression in the *Ripply2*$^{-/-}$ embryo.

Mesp2, but not Lfng, is responsible for the Notch suppression necessary for rostro-caudal patterning

In somite-stage embryos, Notch activity oscillates in the posterior PSM and stabilizes as a clear stripe in the anterior PSM with elevated activity (Huppert et al., 2005; Morimoto et al., 2005). To further understand the molecular events operating in the anterior PSM of *Ripply2*$^{-/-}$ embryos, we first examined the expression of *Notch1* mRNA (Fig. 6A,B) and Notch1 protein (Fig. 6C,D) in these embryos. Interestingly, these expression patterns were found to be expanded in the anterior PSM in the *Ripply2*$^{-/-}$ embryo (Fig. 6B,D), but the Notch activity appeared to be lost as judged from the fact that the expression of *Hes5*, a Notch target gene (Ohtsuka et al., 1999), was absent (Fig. 6E,F). To further confirm this reduced Notch1 activity and its relationship to Mesp2 expression, we conducted double immunostaining analysis using anti-active Notch1 and anti-Mesp2 antibodies in both wild-type and *Ripply2*$^{-/-}$ embryos. In the wild-type embryos, the Notch activity in the anterior PSM exhibited a sharp boundary with Mesp2 that determines the next segmental boundary (Fig. 6G). In addition, the contrast between Notch activities leads to the generation of future rostral and caudal compartments of the somites, whereby the Notch active site becomes the future caudal compartment. In the *Ripply2*$^{-/-}$ embryo, the Notch1 signals oscillated normally in the posterior PSM (Fig. 6H,I). However, the elevation of Notch activity in the anterior PSM appeared to be repressed in these null embryos, whereas the Mesp2 expression banding was found to upregulated, as shown previously (Fig. 6H,I).

Since the expression of *Lfng* is under the control of Mesp2, we speculated that the suppression of Notch signaling might be the result of the prolonged activation of *Lfng* in the *Ripply2*$^{-/-}$ embryo. To test this possibility, we generated a *Ripply2/Lfng* double-knockout embryo from which we prepared skeletal specimens, and then examined the somite properties by analyzing the expression of the caudal molecular marker *Uncx4.1* (Fig. 7). Intriguingly, the vertebral morphology of the *Ripply2/Lfng*

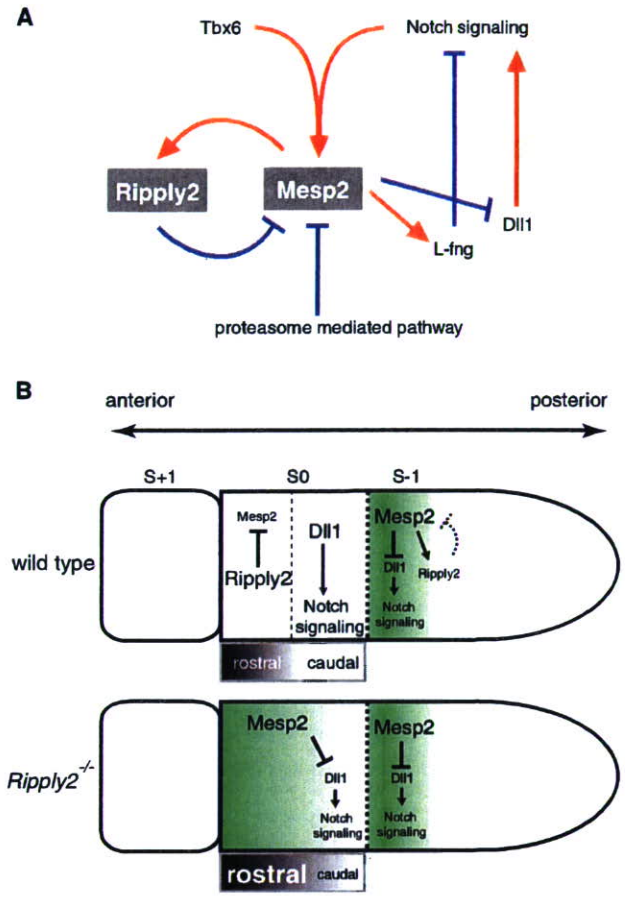

Fig. 8. Genetic cascades in the anterior PSM regulating somitogenesis. (**A**) Schematic of the positive (red line) and negative (blue line) regulation surrounding Mesp2. The transcription of *Mesp2* is enhanced by both Notch signaling and Tbx6. At the same time, Mesp2 suppresses Notch signaling by activating *Lfng* and suppressing *Dll1* expression. Mesp2 proteins are also rapidly degraded via a proteasome-dependent pathway. We herein propose a new negative regulatory system for Mesp2 via Ripply2. (**B**) Schematic illustrating how the rostro-caudal polarity is established or disrupted in the anterior PSM of the wild type and *Ripply2*$^{-/-}$ mutants. In the anterior PSM, Mesp2 is localized in the rostral compartment of S–1 and suppresses Notch signaling through the suppression of *Dll1*. By contrast, in the caudal compartment of S0, both *Dll1* expression and Notch signaling are retained because of the lack of Mesp2. In the *Ripply2*$^{-/-}$ embryo, Mesp2 expression persists for a longer period in both the rostral and caudal compartments, although the suppression on Notch signaling is incomplete. This results in the expansion of the rostral properties within the somites.

double-knockout mouse was not recovered, and was more rostralized as compared with either the *Ripply2*$^{-/-}$ (compare Fig. 4F with Fig. 7C) or *Lfng*-null fetus (Fig. 7B). The expression of *Uncx4.1* was also not recovered by the additional loss of Lfng (compare Fig. 5B with Fig. 7C), and was found to be completely diminished in the double-knockout embryos.

To determine whether the suppression of Notch signaling is mainly due to the function of Mesp2, we also generated *Mesp2/Ripply2* double-null mice and analyzed the resulting skeletal phenotypes. As expected, the vertebral morphology of these fetuses was found to be very similar to the *Mesp2* single-null fetus, and exhibited a caudalized phenotype (Fig. 7D,E). The expression of *Uncx4.1* was also upregulated to similar levels as in the *Mesp2*-null embryo (Fig. 7E). These results clearly showed that Mesp2 suppresses the expression of this gene independent of Ripply2, and that the defect observed in the *Ripply2*$^{-/-}$ mouse can be attributed to the function of Mesp2.

DISCUSSION

Our current study establishes the hypothesis that the negative-feedback regulation of Mesp2 by Ripply2 constitutes a core component of the regulatory network involved in establishing rostro-caudal patterning. The periodicity of somitogenesis is established by mechanisms based on the negative regulation of several genes in the mouse posterior PSM (Rida et al., 2004), in which the clock genes *Hes7* and *Lfng* are negatively regulated by several mechanisms, including transcriptional suppression, protein degradation and destabilization of mRNA (Bessho et al., 2003; Chen et al., 2005; Cole et al., 2002; Hirata et al., 2004; Morales et al., 2002). In the anterior PSM, the levels of Mesp2 are strictly regulated to achieve the periodic suppression of Notch signaling, and also to establish the correct rostro-caudal polarity. During this activation step, the cooperation between Tbx6 and cyclic activated Notch-signaling is crucial for the periodic induction of *Mesp2* (Yasuhiko et al., 2006) (Fig. 8A). However, these processes must be regulated by both activation and inhibition. Previously, we reported that Mesp2 is regulated negatively by the proteasome pathway (Morimoto et al., 2006). In addition, our current study has identified Ripply2 as a potent negative regulator of *Mesp2* transcription, and as a factor that is required for the correct establishment of rostro-caudal patterning. In the absence of Ripply2, Mesp2 expression is maintained over a longer period and leads to the suppression of caudal properties (Fig. 8B). It is noteworthy in this regard that Ripply2 might function exclusively to negatively regulate Mesp2, because the phenotype of the *Ripply2*-knockout mouse is almost completely reversed by the additional loss of Mesp2.

The *Ripply2*-null mutant exhibits not only an expansion of rostral marker genes but also a reduction in the expression of caudal markers. Immunohistochemical analysis further revealed a decrease in the activated form of Notch1 in the anterior PSM in these *Ripply2*-null embryos. Previously, we have shown that Mesp2 suppresses Notch signaling to establish segmental boundaries via the activation of Lfng. However, Lfng appears not to be crucial for the suppression of Notch signaling in the *Ripply2*-null embryo as this suppression was not rescued by the additional loss of Lfng, and, in fact, this results in a further reduction in Notch signaling activity. We speculate that this is caused by the function of Lfng during Mesp2 distribution, based upon our observations of the Mesp2-venus knock-in mouse. In the wild-type embryo, the Mesp2-venus expression pattern shows a clear gradient, being higher in the presumptive rostral compartment. However, in the absence of Lfng, such a biased gradient is not generated, and the Mesp2-venus pattern shows a diffuse distribution in this background (our unpublished data). The phenotype of the *Ripply2/Lfng* double-knockout mouse appears also to reflect this distribution defect. In this double-null mouse, the expression of Mesp2 is prolonged owing to the lack of Ripply2, and is distributed across a much wider area along the anterior-posterior axis because of the lack of Lfng. This in turn enhances the function of Mesp2 that suppresses Notch signaling in the anterior PSM, and results in the somites becoming completely rostralized in these double mutants.

The mechanisms underlying the suppression of Mesp2 by Ripply2 are currently unknown. Ripply2 appears to be required for the termination of Mesp2 expression at an appropriate time. Moreover, because Ripply2 has no apparent DNA-binding domain, it is plausible to assume that it suppresses *Mesp2* by recruiting the Groucho homolog Tle1 and/or Tle3 via the WRPW motif, as revealed previously by in vitro assays in both zebrafish and mouse (Kawamura et al., 2005) (data not shown). *Tle1* and *Tle3* are known to be expressed in the PSM, but their expression patterns are not segmental (Dehni et al., 1995) (our unpublished data), and no loss-of-function studies have yet been reported. In the zebrafish, *ripply1* morphants also display upregulation of *mespb* in their somitic regions, and this is accompanied by the upregulation of *tbx24*, *deltaC* and *deltaD*. This might also account for the upregulation of *mespb* (Kawamura et al., 2005). We have previously identified a 300 bp upstream region of the *Mesp2* gene as a promoter-enhancer sequence required for the faithful expression of *Mesp2* in the anterior PSM where T-box factor binding in combination with Notch signaling is involved in the gene activation (Yasuhiko et al., 2006). However, Tbx6 expression is unchanged (data not shown) and the *Dll1* expression profile is somewhat decreased in the *Ripply2*-null embryos. Hence, although the impact of the loss of Ripply proteins upon Mesp gene expression appears to be similar between mouse and zebrafish, the underlying mechanisms might well be different.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/8/1561/DC1

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Gene Expression Profiles in T24 Human Bladder Carcinoma Cells by Inhibiting an L-type Amino Acid Transporter, LAT1

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Inhibition of LAT1 (L-type amino acid transporter 1) activity in tumor cells could be effective in the inhibition of tumor cell growth by depriving tumor cells of essential amino acids. Because of the high level of expression of LAT1 in tumor cells, LAT1 inhibitors would be useful for anticancer therapy in suppressing tumor growth without affecting normal tissues. In recent years, cDNA microarray technique is useful technology for anticancer drug development. It allows identifying and characterizing new targets for developments in cancer drug therapy through the understanding genes involved in drug action. The present study was designed to investigate gene expression profile induced by LAT1 inhibitor using gene chip technology. Human bladder carcinoma cells (T24 cells) were treated with classical system L inhibitor 2-aminobicyclo-(2, 2, 1)-heptane-2-carboxylic acid (BCH). Gene chip experiment was applied for treated and untreated cells after 3 and 12 h. Two independent experiments with a high degree of concordance identified the altered expression of 151 and 200 genes after 3 and 12 h BCH treatment. Among these genes, 132 and 13 were up-regulated and 19 and 187 were down-regulated by 3 and 12 h BCH treatment respectively. We found that BCH affected the expression of a large number of genes that are related to the control of cell survival and physiologic behaviors. These data are useful for understanding of intracellular signaling of cell growth inhibition induced by LAT1 inhibitors as candidate for anticancer drug therapy.

Key words: BCH, Gene expression, Microarray, Bladder carcinoma cells, LAT1

INTRODUCTION

System L is a major nutrient transport system responsible for the Na^+-independent transport of large neutral amino acids including several essential amino acids (Christensen, 1990; Oxender and Christensen, 1963). In malignant tumors, a system L transporter L-type amino acid transporter 1 (LAT1) is up-regulated to support tumor cell growth (Kanai *et al.*, 1998; Sang *et al.*, 1995; Wolf *et al.*, 1996; Yanagida *et al.*, 2001) . It is proposed that the manipulation of system L activity, in particular that of LAT1, would have therapeutic implications. The inhibition of LAT1 activity in tumor cells could be effective in the inhibition of tumor cell growth by depriving tumor cells of essential amino acids (Kanai and Endou, 2001). Our previous studies have revealed that T24 cells express LAT1 in the plasma membrane together with its associating protein 4F2hc that inhibited by a system L-specific inhibitor 2-aminobicyclo-(2, 2, 1)-heptane-2-carboxylic acid (BCH) (Kim *et al.*, 2002; Yanagida *et al.*, 2001). An increased understanding of molecular mechanisms of LAT1 inhibitors will lead to the development LAT1 inhibitors for anticancer drug therapy.

cDNA microarray analysis permits the simultaneous and rapid analysis of the expression of tens of thousands of genes, and, in turn, provides an opportunity for determining the effects of anticancer agents. This technology will contribute to the more accurate development of therapeutic strategies and will help to determine the molecular mechanism(s) of action of chemopreventive and/or therapeutic agents (Li and Sarkar 2002; Macgregor and Squire,

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2002). To better understand the precise molecular mechanisms by which BCH exerts its effects on T24 bladder carcinoma cells, we utilized a cDNA microarray to interrogate the mRNA levels of 39,000 genes and to determine the gene expression profiles of T24 bladder carcinoma cells treated with BCH.

MATERIALS AND METHODS

Cell culture and growth inhibition

T24 human bladder cancer cells were cultured in the growth medium (minimum essential medium supplemented with 10% fetal bovine serum) in a 5% CO_2 atmosphere at 37°C. For growth inhibition, 60 h after seeding (during logarithmic phase) T24 cells were treated with 20 mM BCH for 3 and 12 h.

cDNA microarray analysis for gene expression profiles

T24 cells were treated with 20 mM BCH for 3 and 12 h. The rationale for choosing these time points was to capture gene expression profiles of early response genes, genes that may be involved during the onset of growth inhibition. Total RNA from each sample was isolated by Trizol (Invitrogen) and purified by RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufactures' protocols. cDNA for each sample was synthesized by using Superscript cDNA Synthesis Kit (Invitrogen) using T7-(dT)24 primer instead of the oligo (dT) provided in the kit. The biotin-labeled cRNA was transcripted in vitro from cDNA using a BioArray HighYield RNA Transcript Labeling Kit (ENZO Biochem, New York, NY) and purified using an RNeasy Mini Kit. The purified cRNA was fragmented by incubation in fragmentation buffer (200 mmol/L Tris-acetate pH 8.1, 500 mmol/L KOAc, 150 mmol/L MgOAc) at 95°C for 35 min and chilled on ice. The fragmented labeled cRNA was applied to Human Genome U133 Array (Affymetrix, Santa Clara, CA), which contains 39,000 human gene cDNA probes, and hybridized to the probes in the array. After washing and staining, the arrays were scanned using a HP GeneArray Scanner (Hewlett-Packard, Palo Alto, CA). Two independent experiments were performed to verify the reproducibility of results.

Microarray data normalization and analysis

Data analysis was performed with the GeneChip Expression Analysis software (Affymetrix) and GeneSpring TM software (Silicon Genetics, Redwood, CA, U.S.A.). The differences in hybridization efficiency among arrays were equalized by intensities of spiked-in control mRNAs added to the sample in proportion to its DNA content (Kanno *et al.*, 2006). In a set of GeneChip experiments comparing the same sample hybridized to two different arrays, method-associated experimental artifacts produced less than two-fold differences between two identical samples. Thus, genes displayed over two-fold expression change were subjected to further testing. GeneChip array HG-U133A represents 22283 transcripts and the genes whose absolute call judged "present" in at least 1 sample were used for further analysis.

RESULTS

Cell growth inhibition by BCH treatment

Cell count showed that the treatment of T24 bladder cancer cells with BCH, time dependently inhibited cell proliferation (Fig. 1), demonstrating the growth inhibitory effect of BCH. These results are consistent with our previous study. This inhibition of cell proliferation could be due to altered regulation of gene expression by BCH.

Regulation of gene expression by BCH treatment

The gene expression profiles of T24 cells treated with BCH were assessed using cDNA microarray. Two inde-

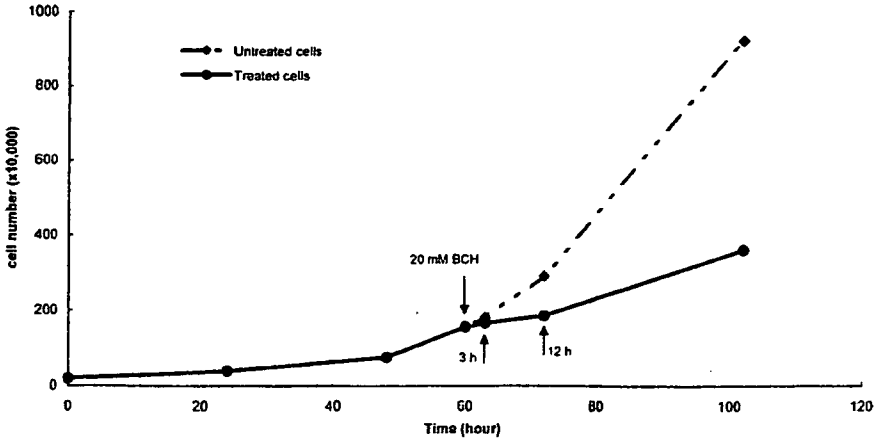

Fig. 1. T24 cell growth curve. BCH was added to treated cells 60 h after seeding.